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THE EFFECTS OF PYRIDOSTIGMINE AND PHYSOSTIGMINE ON THE CHOLINERGIC SYNAPSE

ANNUAL/FINAL REPORT

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19. Abstract (continued)

cholinesterase (ChE) depression of 60-70% exhibited alterations in all three muscle fiber types. These lesions appeared similar in severity, indicating that pyridostigmine does not selectiveldy affect one muscle fiber type at this level of ChE depression. 2) The alterations appeared similar in nature and extent following drug exposure by single injection and by osmotic minipump. Thus, at 60-70% ChE depression, the method of drug administration did not affect the severity of the pathology. 3) At least some NMJs of all three muscles underwent a period of partial denervation following acute (ChE depression approximately 90%) or subacute (ChE depression approximately 70%) pyridostigmine exposure. However, recovery was in process and sometimes complete 60 days following drug exposure, indicating that pyridostigmine-induced alterations are reversible. Following a 90-day recovery period, significant reversal of damage to presynaptic organelles and postsynaptic organelles and myofibrils was noted. However, presynaptically, some localized separations of nerve terminals and junctional folds remained. This is a general indication of a high level of metabolic activity. Whether these remaining morphological changes represent a physiological state significantly different from that of control fibers is not known.

SUMMARY

The quaternary carbamate pyridostigmine bromide has been suggested for use in prophylaxis against intoxication with irreversible cholinesterase inhibitors. Since virtually no anatomical data were available concerning the neuromuscular toxicity of the drug, a study was undertaken to evaluate the effects of acute and subacute of pyridostigmine exposure on the ultrastructure of rat diaphragm neuromuscular junctions (NMJs) and muscle fibers. The results of these investigations were reported in the Annual Reports for Years 1 and 2 of contract DAMD-17-83-C-3126. The data suggested a dose-dependent effect of pyridostigmine which resulted in both pre- and postsynaptic alterations of the diaphragm NMJs. The investigations to included analyses of the effects of different modes of pyridostigmine administration on three muscles (diaphragm, extensor digitorum longus [EDL] and soleus). In addition, physiological recovery processes, as reflected by morphological changes, were assessed during extended periods following drug exposure. The results of these studies are summarized below and detailed in the Methods, Results, and Discussion sections of this report.

Neuromuscular junctions from diaphragm, soleus and EDL muscles of male albino rats were assessed for morphological alterations following acute (30 minute) and subacute (2-14 day) exposure to pyridostigmine and during postexposure recovery periods of up to 90 days. The experiments were designed to provide data necessary to accomplish three goals which were: (1) to compare the effects of drug administration by single injection and by osmotic minipump (continuous infusion), (2) to determine whether pyridostigmine selectively affects fast or slow twitch muscle fibers, and (3) to monitor and evaluate morphological changes during long-term recovery.

The diaphragm, soleus and EDL muscles were selected to compare the effects of the method of drug administration (injection versus infusion) on muscles of different fiber type composition. The diaphragm has approximately equal numbers of type I and type II fibers, while the soleus and EDL possess primarily type I and type II fibers, respectively. Pyridostigmine was administered to each acute exposure animal by a single subcutaneous injection of 0.36 mg/kg pyridostigmine and to each subacute exposure animal by a subcutaneously implanted osmotic minipump containing 10 mg/ml pyridostigmine. Both treatments resulted in a whole blood cholinesterase (ChE) depression of approximately 60-70% as determined by radiometric assay. Control animals received only Mestinon-equivalent diluent.

Both acute and subacute exposures resulted in morphological alterations of the NMJs of all three muscles,

although considerable variation in the extent of damage occurred even within individual NMJs. The most frequently observed presynaptic alterations were mitochondrial damage and partial withdrawal of nerve terminal branches (partial denervation). Postsynaptic changes included occasional rarefaction of mitochondrial matrices and disruption of the myofibrillar organization in small numbers of subjunctional sarcomeres. The data indicate that acute or subacute exposure to pyridostigmine bromide at a whole blood ChE depression of 60-70% results in similar alterations to the NMJs of the three muscles with substantially different fiber type populations. At this dose level, the severity of the damage varied from fiber-to-fiber in an apparently random manner and did not appear to be related to a specific fiber type or dosage regimen.

Long term recovery processes were evaluated following exposure to 1.0 mg/kg pyridostigmine by single injection and following 14 days of exposure to pyridostigmine by osmotic minipump (total dose 20 mg pyridostigmine). Recovery was analyzed at 7, 14, 21, 35, 60 and 90 days following withdrawal from drug. Whole blood cholineterase activity levels were monitored by radiometric assay before, during and periodically following drug exposure. Following a 30 minute exposure to 1.0 mg/kg, ChE levels were approximately 10% of preinjection values. Animals exposed to 10 mg/ml pyridostigmine maintained a relatively constant ChE activity of approximately 30% throughout the 14 day exposure period. Preliminary data indicate that postexposure ChE values initially exceeded pre-exposure ChE levels and finally approximated control values by 90 days recovery.

At the dose levels studied, NMJs of all three muscles initially underwent the typical pre- and postsynaptic alterations described above. As previously reported, signs of partial denervation increased during the 35 day period following pyridostigmine exposure by single injection or minipump. However, indications of terminal sprouting were also apparent by day 21 of the recovery period. Although some pre- and postsynaptic morphological irregularities persisted at day 60 of recovery, the general trend appeared to be toward recovery of pre-exposure morphology in all three muscles. Following 90 days of drug-free recovery, significant reversal of damage to presynaptic organelles and postsynaptic organelles and myofibrils was noted. However, presynaptically, some localized separations of nerve terminals and junctional folds remained. This is indicative of decreased synaptic efficiency. Postsynaptically, numerous nuclei exhibited invaginations often associated with Golgi, polyribosomes and endoplasmic reticulum. This is a general indication of a high level of metabolic activity. Whether these remaining morphological changes represent a physiological state significanly different from that of control fibers is not known.

FOREWORD

Citations of commercial organizations and trade names in this report do not constitute an official Department of the Army endorsement or approval of the products or services of these organizations.

In conducting the research described in this report, the investigator adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 78-23, Revised 1978).

TABLE OF CONTENTS

STATEMENT OF PROBLEM

Pyridostigmine bromide, a reversible anticholinesterase drug, has been suggested for use in prophylaxis against intoxication with irreversible cholinesterase (ChE) inhibitors. Recent investigations have indicated that in vivo exposure to the drug results in morphological alterations to the neuromuscular junctions (NMJs) of the rat diaphragm muscle (Hudson and Foster, 1984; Hudson, et al., 1985a, 1985b). In these studies, acute doses of pyridostigmine bromide in Mestinon-equivalent buffer were administered by single subcutaneous injection in doses that ranged from 0.0036 to 3.6 mg/kg (0.001-1.0 LD50). Tissues were analyzed in the acute experiments at 10-30 minutes, and 2 and 7 days postinjection. Subacute exposures were administered by subcutaneously implanted osmotic minipumps that contained either 3.0 or 20 mg pyridostigmine in Mestinon-equivalent buffer. Tissues were analyzed after subacute exposures of 3, 7 or 14 days and after postexposure recovery times of 7, 14 or 23 days. In selected animals, whole blood ChE activity was measured by radiometric assay. The morphological effects of the drug were localized predominantly at the NMJ with both pre- and postynaptic regions involved.

The data indicated that there was variation in the extent of the damage between different muscle fibers and between different areas of an individual NMJ. With acute doses, there was a dose-dependent response which was manifest in terms of the location of the pathology. Low doses seemed to affect the presynaptic area with no apparent effects on the muscle cell. At higher doses (0.01-1.0 LD50), both pre- and postsynaptic elements became involved.

Subacute exposure also caused dose-dependent lesions in the diaphragm. At 14 days, the 20 mg dose group exhibited pre-and postsynaptic alterations which were more extensive than those seen in the 3 mg group although neither dose caused damage equivalent to that seen 30 minutes after a single LD50 dose. Presynaptic alterations included withdrawal of the terminal from the junctional folds, invasion of the synaptic cleft with Schwann cell processes, and disruption of axon terminal organelles. Postsynaptic alterations included subjunctional supercontraction, disruption of myofibrillar apparatus and disruption of subjunctional mitochondria. Ultrastructural recovery of the diaphragm after acute or subacute exposure to the drug varied. The evidence suggested that subacute exposure had the greatest effect within the first week of exposure, with additional exposure sustaining but not appreciably increasing the extent of the lesion. Diaphragms subjected to moderate acute doses exhibited a progression in the degenerative process (including partial denervation) for at least one week

postexposure. Evidence of recovery from the drug-induced effects included reinnervation of junctional areas and the replacement or repair of subcellular elements.

This study was designed to accomplish the following:

a. To establish subjectively whether pyridostigmine exposure by single injection induced more extensive damage than exposure by osmotic minipump when both methods resulted in similar ChE depressions.

b. To determine whether pyridostigmine selectively damages one fiber type more severly than another.

c. To assess the recovery processes in pyridostigmine-exposed diaphragm, extensor digitorum longus (EDL) and soleus NMJs to determine whether the three muscles recover in a similar manner and time frame.

d. To develop appropriate freezing and cryosectioning techniques for studying subjunctional ion distributions in control and pyridostigmine-exposed muscle fibers.

BACKGROUND

Pyridostigmine is an anticholinesterase drug capable of rapidly inhibiting acetylcholinesterase in mammals by covalent enzyme carbamylation. Clinically, in its Mestinon formulation, the drug is used primarily in the treatment of myasthenia gravis. Experimentally, when combined with cholinolytic and oxime therapy, pyridostigmine has been shown to be an effective prophylactic against systemic exposure to irreverisible organophosphate ChE inhibitors. Pyridostigmine prophylaxis is due, presumably, to the continuing production of a noninhibited pool of acetylcholinesterase which is derived from the spontaneous decarbamylation of the previously pyridostigmine-inhibited enzyme (Barry and Davies, 1970; Gordon, et al., 1978; Dirnhuber, et al., 1979).

Numerous reports have established that the irreversible ChE inhibitors produce myopathies and/or neuropathies and are responsible for abnormal physiology at the mammalian NMJ (Preusser, 1967; Ariens, et al., 1969; Fenichel, et al., 1972, 1074; Laskowski, et al., 1975; Wecker and Dettbarn, 1976; Wecker, et al., 1979; Salpeter, et al., 1979, 1982). Until recently, the quaternary carbamate neostigmine was the only carbamate anticholinesterase drug employed in anatomical studies to evaluate the potential toxicity of this class of drugs vis-a-vis neuromuscular pathology (Engel, et al., 1973; Engel and Santa, 1973; Ward, et al., 1975; Hudson, etal., 1978). The paucity of anatomical data available concerning pyridostigmine-induced neuromuscular alterations prompted our recent ultrastructural study which assessed presynaptic damage resulting from pyridostigmine exposures (Hudson, et al., 1985a, 1985b). The data revealed that acute or subacute administration of pyridostigmine resulted in withdrawal of localized portions of the nerve terminal from the junctional folds, invasion of the synaptic cleft by Schwann cell processes and disruption of nerve terminal organelles (Hudson, et al., 1985a, 1985b). Postsynaptic changes included disruption of myofibrillar organization and damage to membrane-bound organelles (Hudson, 1988). The severity of both pre- and postsynaptic alterations was dose dependent.

These results indicated the necessity to extend the investigations to establish additional information concerning the ultrastructural effects of pyridostigmine. Thus, experiments were designed to determine whether drug administration by single injection induced similar or more severe NMJ pathology than drug administration by continuous infusion (osmotic minipump) when comparable ChE depressions were maintained. Second, the NMJ alterations were compared in the diaphragm, EDL and soleus to establish if pyridostigmine selectively affects one fiber type more severely

than another. Third, NMJs of all three muscles were monitored to determine the extent of the recovery processes which occurr following pyridostigmine exposure. Fourth, the technology was developed to assess localized changes in subjunctional ion distributions as determined by x-ray microanalysis.

MATERIALS AND METHODS

Male albino rats (Edgewood or Charles River strains) weighing 180 to 250 g received subcutaneous, acute and subacute exposures to pyridostigmine bromide. All acute exposures were by single syringe injections under the skin of the midback region and all subacute exposures (maximum 14 days) were via osmotic minipump (Alzet 2ML2 minipump; ALZA Corp., Palo Alto, CA.) implanted under the skin of the midback. Pyridostigmine bromide was administered in a Mestinon -equivalent diluent composed of 1.30 mg/ml citric acid monohydrate, 4.10 mg/ml sodium citrate dihydrate, 0.50 mg/ml methyl paraben, 0.05 mg/ml propyl paraben and 7.40 mg/ml sodium chloride in sterile water at pH 5.1.

Acute Drug Exposure. Acute doses of pyridostigmine ranged from 0.0036 mg/kg to 3.6 mg/kg (LD)subcutaneous injection = 3.6 mg/kg determined by probit analysis). Animals in the acute control group received a single injection of the Mestinon -equivalent diluent. A minimum of 3 experimental and 2 control animals were prepared for each dose level analyzed. These animals were sacrificed under deep barbiturate anesthesia by transcardiac vascular perfusion at 10-30 minutes, and 2, 7, 21, 35, 60, and 90 days postinjection. These time periods allowed evaluation of acute drug effects as well as short and long term recovery processes.

Subacute Drug Exposure. Subacutely treated animals had 2 ml osmotic minipumps implanted subcutaneously to provide continuous infusion of pyridostigmine. Use of osmotic minipumps allowed continuous release of the drug in order to maintain both tissue-ChE activity and tissue-drug levels as constant as possible.

Subacute doses were studied using minipumps loaded with 10.0 mg/ml pyridostigmine bromide in the Mestinon-equivalent buffer. Control animals were implanted with minipumps containing the Mestinon-equivalent buffer. The morphological effects of chronic infusion of pyridostigmine were evaluated after exposure periods of 2, 7 and 14 days. The processes of recovery from subacute pyridostigmine exposure were assessed at 7, 14, 21, 35, and 60 days after a 14-day exposure by minipump. A minimum of 3 experimental and 2 control animals were prepared and analyzed for each dose at each exposure period. Since the Alzet pumps utilized in this study released their contents at a rate of approximately 5.9 ul/hr, the experimental animals were exposed to an approximate total of 2.8, 10 and 20 mg of drug on 2, 7 and 14 days, respectively. Blood ChE activity of each drug-treated and control animal were analyzed (Siakotos et al., 1969 see below) periodically (at least twice) during the course of the experiment.

Preparation for Electron Microscopy. All animals were prepared by whole body perfusion through the left ventricle using an initial perfusate of rat Ringer's solution containing 5mM KCL, 1mM MgCL, 2mM CaCl, 15mM NaHCO, 1mM Na HPO and 10 units/ml of heparin followed by the fixation perfusate containing 2.5% glutaraldehyde in 0.1M sodium cacodylate buffer (pH 7.4). The perfusates were maintained at room temperature. The diaphragm and both soleus and EDL muscles of each animal were immediately removed and fixed for an additional hour in cold 2.5% glutaraldehyde. NMJs were identified by staining for ChE activity in a solution of 5 mg acetylthiocholine iodide, 6.5 ml 0.1 M sodium cacodylate buffer (pH 7.4) with 0.2 M sucrose, 0.5 ml 0.1 M sodium citrate, 1.0 ml 30 mM copper sulfate and 1.0 ml ddH 0 (modified from Karnovsky and Roots, 1964). Endplate regions and tissue remote from the endplates were removed by careful dissection, postfixed in 1% OsO , stained en bloc in aqueous uranly acetate or 2% uranyl acetate in 70% ethyl alcohol embedded in an Epon-Araldite mixture (10% Poly-bed 812, 20% Araldite 70 C for 24 hours. Ultrathin sections were cut with a diamond knife, using an LKB III or IV ultra-microtome, poststained with lead citrate (Venable and Coggeshall, 1965) and aqueous or methanolic uranyl acetate and examined with a JEOL 100 CX electron microscope.

Cholinesterase Assay. The radiometric method of Siakotos et al. (1969) using 14C-acetylcholine as the ChE substrate was utilized throughout. The relative ChE depression in whole blood produced by acute or subacute exposure to pyridostigmine was determined. Blood (100 ul) was drawn from the tail into heparinized capillary tubes and processed for an immediate assay or frozen in liquid nitrogen for subsequent assay. In order to test for the effects of freezing whole blood on the assay results, the 100 ul blood was divided into two 50 ul aliquots, one used for immediate assay and the other frozen for later assay. Freezing prior to assay resulted in a variation of approximately 5% of the ChE depression established by immediate assay. This was within an acceptable range for the purpose of the present study.

The enzyme assay was performed in the following manner. Up to 100 ul usually 5-50 ul) of blood sample was added to 100 ul 0.1M sodium phosphate (pH 7.4), 10 ul MgCl and 100 ul 14C-acetylcholine. The solution was mixed immediately and incubated for 5 minutes at 37 C (with shaking). The reaction was stopped by adding sufficient resin-dioxane mixture (20g Amberlite CG - 120 x 8 and 100 ml dioxane) to increase the total volume to 5 ml. The enzymeresin-dioxane volume was then brought to 10 ml with dioxane, mixed by inversion (3 times) and centrifuged at 900g for 3 minutes. Then 1.0 ml of supernatant was combined with 10 ml of cocktail and the radio-activity assayed in a Beckman LS-7800 liquid

scintillation counter. A reagent blank was run simultaneously ,using water in place of sample.

Since it was not practical to gather the ChE activity values for all of the acutely-treated animals, a parallel study (using a group of rats not utilized in the morphological study) was employed to establish some of the data for a dose-response curve of enzyme carbamylation at the time point of 30 minutes postinjection (single subcutaneous). Animals in the recovery studies were assessed for whole blood ChE levels periodically throughout the 90-day period following exposure by single injection. A minimum of three drug-treated animals and one control animal was used for the determination of total whole blood ChE activity at each dose.

For the animals in the subacute exposure groups, ChE levels were assayed before and periodically after the implantation of the osmotic minipump. The assay was performed on these animals (a) to confirm that each minipump was releasing the dr[illegible], (b) to establish the general dose/ChE activity relationship for each dose and each animal and (c) to determine the recovery of ChE activity levels during the 90-day period following exposure by continuous infusion.

RESULTS

General Observation. Animals in the acute and subacute treatment groups were monitored for behavioral signs of pyridostigmine intoxication periodically during the period of drug exposure. Animals in the acute exposure group were assessed for outward behavior responses 30 minutes following injection of pyridostigmine or diluent. The behavior of animals in the recovery studies was also regularly evaluated throughout the postexposure period. No obvious signs of anticholinesterase intoxication were detected in any of the animals in this acute exposure study.

Similarly, animals implanted with osmotic pumps containing pyridostigmine or diluent exhibited no detectable behavioral signs of pyridostigmine intoxication over the 14 day period of exposure or during subsequent recovery periods of up to 90 days with the exception of 4 animals. These 4 of 50 rats exhibited increased ocular secretions (chromodacryorrhea) within the initial few hours of pyridostigmine exposure but displayed no other signs of anticholinesterase intoxication. The chromodacryorrhea cleared within the first 24 hours of exposure and did not reappear. These animals as well as the rest of the experimental animals remained symptom-free for the duration of the experiment.

Whole Blood Activity Animals were exposed to various doses of pyridostigmine to determine the average inhibition (enzyme carbamylation) of whole blood ChE activity after 30 minutes. The data are presented in Figure 1. Doses of 0.0036 and 0.006 mg/kg pyridostigmine resulted in ChE levels within the control range. Thus, these doses either had no significant effect on the whole blood ChE activity or were at the limit of sensitivity of this ChE assay. However, larger doses resulted in significantly decreased ChE activities at the 30 minute endpoint. The data indicate a clear dose dependence at the higher drug concentrations. The effect of pyridostigmine on ChE activity virtually plateaus between 1.0 mg/kg and 3.6 mg/kg.

The whole blood Che levels of subacutely treated animals are presented in Figure 2. Again the data reflect the relationship of the ChE level to the pyridostigmine dose. Following 2 days of drug exposure, the ChE activity of animals with low dose pumps was approximately 70% of control values. In this group of experimental animals, the mean ChE activity was within the range of control values at day 14 of exposure. In the animals with high dose pumps, a continuous enzyme inhibition was maintained. ChE activities, in this group, ranged between 30% to 40% of control values throughout the period of drug exposure. Thus, the whole blood ChE activity decreased during the initial 24 to 48 hour period

following minipump implantation. Prolonged exposure to the drug did not produce additional significant decreases in enzyme activity.

The whole blood ChE activity was monitored at intervals up to 90 days followign single injections of 0.036 mg/kg pyridostigmine and 1.0 mg/kg pyridostigmine (Figure 3). Recovery of the ChE activity occurred following both doses and remained within control levels through the 90 day recovery period.

The whole blood ChE activity was monitored at intervals up to 90 days following continuous infusion of 1.5 mg/ml and 10.0 mg/ml pyridostigmine via 2 ml osmotic minipumps (Figure 4). Recovery from drug-induced decreases in ChE activity occurred following both doses.

ULTRASTRUCTURAL OBSERVATIONS

Neuromuscular Junction Morphology of Control Animals

Control animals were either (1) untreated, (2) received a single injection Mestinon-equivalent diluent, or (3) were implanted with osmotic minipumps containing the Mestinon-equivalent diluent. Ultrastructural morphology of NMJs from untreated rats and from rats treated with diluent appeared similar (Figs. 5,6,7). Nerve terminals contained numerous synaptic vesicles and variable numbers of mitochondrial profiles. The nonsynaptic surfaces of the nerve terminals were in close apposition to Schwann cells (Figs. 5,6,7) which possessed processes that extended to the margins of the primary cleft but not usually into the cleft. Typically, a 50 nm wide primary cleft containing basement membrane separated the pre- and postsynaptic components. Postsynaptically, the sarcolemma was differentiated into a series of junctional folds which were separated from each other by 50-100 nm secondary clefts. The sarcoplasm of the endplate region usually contained one to several nuclei, numerous mitochondria, Golgi, polyribosomes and rough endoplasmic reticulum cisternae. All of these organelles were not present in every section of a NMJ but were distributed throughout the endplate region. Rest-length sarcomeres were characterized by a regular pattern of Z lines, and A, H and I bands. Thus, analysis of NMJs from control animals revealed no indications of deleterious effects of the Mestinon-equivalent buffer administered by single injection or by osmotic minipump. NMJs reflect a range of morphological variability due to fiber type, plane of section, position of section within the NMJ (peripheral versus central), state of NMJ turnover, age and inherent individuality. The NMJs in Figures 3, 4 and 5, however, reflect morphological profiles typically observed in control preparations.

The Presynaptic Effects of Acute Exposure to Pyridostigmine. All acute doses employed resulted in morphological alterations ranging from slight to pronounced. In the diaphragm, three muscle fiber types and associated NMJs can be recognized by the ultrastructural criteria of Gauthier and Padykula (Gauthier, 1970; Padykula dn Gauthier, 1970). In this study, fiber type was not determined for each fiber examined. However, at each dose, every NMJ examined possessed similar alterations although the degree of severity sometimes varied. Thus, it does not appear that alterations occurred preferentially in any one fiber type. It is important to keep in mind that 1) different NMJS form the same animal were affected to different degrees and 2) different presynaptic areas within the same NMJ were frequently affected to varying extents. In general, the alterations were observed in mitochondria and synaptic vesicles or in the spatial relationship of the nerve terminal, both to its overlying Schwann cell and to the junctional folds of the innervated muscle cell.

Changes in presyanptic organelle ultrastructure were dose depaendent. Nerve terminals exposed to the lowest acute dose (0.0036 mg/kg) revealed the greatest variability. Mitochondria sometimes possessed small rarefied areas of matrix and infrequently membranes were widely separated from the crests of junctional folds. Occasionlly, Schwann cell projections were present in the primary cleft and localized regions of the nerve terminals were withdrawn from the junctional folds. Similar separations of pre- and postsynaptic elements were observed rarely in control preparations. Most of the NMJs exposed to 0.0036 mg/kg pyridostigmine possessed no or only subtle irregularities in presynaptic morphology. Since a 5% variability in ChE activity and some morpnological variablility within the control range, the physiological relevance of the subtle morphological variability is difficult to evaluate and may be of limited significance to the animal's overall neuromuscular function. At a dose of 0.036 mg/kg, the ChE activity is 50% of control values. At this dose, notable changes were consistantly observed in every nerve terminal analyzed. Mitochondeial alterations generally appeared as small rarefactions in the matrix (Fig.8). Small separations of pre- and postsynaptic elements were occasionally observed while multiple layers of membrane were frequently present within nerve terminals. These sometimes appeared to surround small vesicle-containing portions of nerver terminal (Fig. 8). At higher doses (0.36 mg/kg, 3.6 mg/kg) mitochondrial alterations varied from small rarefactions in the matrix (Fig. 9) to complete disruption of the cristae or absence of the matrix altogether (Figs. 9, 10). The latter, more extreme and probably irreversible alterations were not always observed in terminals, even at the highest doses (3.6 mg/kg; Fig. 12). Vesicles two or more times the average diameter of synaptic vesicles were present, as were occasional vesicles

with denser than normal contents (Figs. 8, 9, 10). Whether these large vesicles represent abnormal synaptic vesicles or originate from another source is unresolved.

The shape and spatial geometry of nerve terminals were also altered following pyridostigmine exposures. In all experimental groups, some of the NMJs samples possessed at least small areas of terminal membrane that were no longer closely opposed to basement membrane. This alteration was usually subtle or absent in NMJs exposed to 0.0036 mg/kg drug. When these regions of separation were present at higher doses, the width of the primary cleft was highly variable and frequently exceeded twice the cleft width observed in control preparatioons. Within the primary synaptic clefts of altered NMJs, finger-like processes (apparently from the Schwann cells) were commonly seen separating areas of the nerve terminal from the postsynaptic folds (Figs. 9, 10). The Schwann cell overlying the nerve terminal remained in close apposition to the nonsynaptic terminal membrane surface (Figs. 8,10-12), but sometimes invaded the terminal with processes (Figs. 8, 9, 11, 12). Fig. 12 illustrates how some invading Schwann processes interdigitate with portions of the nerve terminal. Profiles of both Schwann cell processes and nerve terminal projections appeared finger-like in longitudinal section (Figs. 8, 12). In cross-section, the nerve terminal projections appeared as separate, vesicle-containing, membrane-bound profiles within the nerve terminal or surrounded by a Schwann cell process (Figs. 8, 11). No evidence indicated that the nerve terminal projections were separated from the body of the nerve terminal.

Presynaptic Effects of Subacute Exposure to Pyridostigmine. Neuromuscular junctions of animals implanted with osmotic minipumps containing 1.5 mg/ml or 10 mg/ml pyridostigmine were assessed for damage, 2, 7 and 14 days following implantation of the pumps. Both dose levels induced alterations at all time intervals analyzed. However, the extent of the damage varied with the duration of exposure. In general, the alterations from subacute dosages (Figs. 13-16) were qualitatively similar to the observations in acutely treated rats (Figs. 6-12), i.e., nerve terminal organelles were altered and the spatial geometry of the NMJ was effected. Reminiscent of acute exposures, the extent of nerve terminal damage (Figs. 13-16) from any subacute exposure varied within a single muscle, varied within individual NMJs, and was not confined to one muscle fiber type.

Figs. 13-16 illustrate examples of the organelle alterations resulting from subacute pyridostigmine exposure. Several presynaptic mitochondria possessed rarefied areas in their matrix and nerve terminal vesicles exhibited an unusual range of diameters. The organelle alterations following

subacute exposure never progressed to the degree of severitiy observed following higher doses by acute exposure, i.e., complete disruption of all presynaptic mitochondria was never observed and the numbers of abnormal vesicles appeared lower.

Alteration of the spatial relationship of the nerve terminal and muscle cell was the most obvious effect of subacute drug exposure. The most subtle, observable changes occurred following a low dose esposure to pyridostigmine for two days. This treatment resulted in regional separation of the nerve terminal from the junctional fold crests (Figs. 13, 14). This phenomenon was present in some parat of every NMJ analyzed following two or more days of low or high exposure. In some NMJs, processes of overlyning Schwann cells were interposed between the nerve terminal and the junctional fold crests (Figs. 14, 15), which also resulted in separation of presynaptic transmitting surface from the postsynaptic receptor-containing junctional fold crests. Following 7 and 14 days of subacute exposure, similar alterations were observed. Although quantitation of the severity of changes between low and high dose exposures was not performed, animals exposed to the higher dose of pyridostigmine appeared to possess more obvious and extensive alterations. In general, longer exposure resulted in continued invasion of primary synaptic clefts by Schwann cells (Fig. 15), regional withdrawal of some nerve terminal membranes (Fig. 16), and complete absence (withdrawal) of some nerve terminal portions as evidenced by vacant positions adjacent to postsynaptic folds (Fig. 15). Membrane fragments near the crest of folds indicated degeneration of the missing segments of nerve terminal.

Postsynaptic Effects of Acute Exposure to Pyridostigmine

NMJs from animals which received a single injection of 0.0036 mg/kg pyridostigmine (95% ChE activity) possessed no characteristic drug-induced postsynaptic changes. The lowest dose tested in this study which resulted in alteration of postsynaptic organelles and myofibrils was 0.036 mg/kg pyridostigmine. At this dose, 50% ChE activity remained. Occasional fibers possessed some subjunctional mitochondria with distinct circular, rarefied regions in the matrix and a number of swollen sarcoplasmic reticulum cisternae (not shown). The majority of fibers, however, were similar in appearance to the one in Fig. 17. In these muscle fibers, occasional subjunctional mitochondria possessed rarefied areas of matrix. The margins of these regions of decreased matrix density were distinctly circular in appearance. This subtle change in mitochondrial morphology appears to be the first detectable indication of a drug-related change in the structure of a membrane bound organelle. These rarefied regions are markedly different from the swollen mitochondrial regions sometimes associated with poor fixation. In the latter case, the alterations appears as swelling of the between the outer and inner mitochondrial membranes. Sarcomeres were at rest length and the myofibrillar components appeared unaffected. At this dose, a notable postsynaptic alteration was the occasional presence of subjunctional lamellar bodies (Fig. 17) which were also observed at higher drug doses with no appreciable increase in frequency. These myelin-like structures were sometimes associated with mitochondria or other membrane bound organelles, however, their origin remains unclear. Similar lamellar bodies have been reported in cat neurohypophysis following exposure to the irreversible anticholinesterase agents diisopropylfluorophosphate (DFP) and pinacolylmethyl-phosphonofluoridate (SOMAN; VanMeter, et al., 1985). Presynaptic changes were regularly observed after acute exposure to 0.036 mg/kg of pyridostigmine (Fig. 6; also see Hudson et al., 1986).

All higher acute doses consistently produced subjunctional damage in all fibers analyzed. Following an acute injection of 0.36 mg/kg, mitochondria displayed alterations ranging from circular rarefactions in the matrix (Fig. 18, m1) to extreme dilation of the matrix (Fig. 18, m2). Note that even in the most severely affected mitochondria, the intermembrane space between the outer and inner mitochondrial membranes remained unaffected. It was the compartment within the inner membrane that underwent swelling and damage. In addition to the many altered mitochondria, there remained numerous mitochondria which appeared morphologically unaffected. In general, the most severely damaged organelles were those closest to the base of the junctional folds. Organelle damage tended to decrease with increasing distance from the junctional folds. Swelling of the subjunctional endoplasmic retuculum cisternae and the nuclear envelope was also observed. Nuclear alterations contrasted to those observed in affected mitochondria. The perinuclear space was dilated and the internal nucleoplasm

remained unchanged. At this dose, sarcomeres often lacked distinct striation patterns. Z lines frequently lacked typical uniformity but were distinctly identifiable (not shown). These abnormalities were also pronounced in the immediate subjunctional region but decreased in severity and disappeared with increasing distance from the NMJ.

Acute doses of 2.6 mg/kg (approximately 11 % ChE activity) or greater resulted in dramatic changes in endplate morphology. The most severe postsynaptic damage included gross swelling of the inner mitochondrial compartment. In the most severely affected mitochondria, remnants of matrix were represented by aggregations of flocculant material and cristae were recognizable as irregular vesicular profiles. In the immediate subjunctional region, mitochondrial profiles were 2 to 4 times the diameter of unaffected mitochondria. It is important to note that the space between outer and inner mitochondrial membranes was unchanged even in the most severely damaged organelles. Swelling was also present in other subjunctional membrane-bound organelles and loss of subjunctional sarcomere organization was observed at virtually every NMJ examined. In many fibers, sarcomeres underwent supercontraction and as a result, synaptic components were pushed out of the normal muscle contour, Z line morphology was obliterated and the organized orientation of thick and thin filaments was lost (Fig. 19). These changes to sarcomere structure, as well as those to membrane-bound organelles, were graded with distance from the junction. At the margins of the subjunctional region, disrupted myofibrillar components blended into normal sarcomeres. At these high doses, small regions of disrupted sarcomeres were occasionally observed in nonjunctional portions of muscle cells. Damage of this latter type was observed infrequently. When present, the disorganized sarcomeres blended into normally organized sarcomeres in a manner similar to the graded damage in the immediate area of the NMJ.

Postsynaptic Effects of Subacute Exposure to Pyridostigmine Short term subacute exposure to pyridostigmine via osmotic minipumps containing 1.5 mg/ml resulted in subtle morphological alterations of postsynaptic components. After 2 days exposure (approximately 70% sustained whole blood ChE activity), NMJs were virtually identical to those observed following exposure to acute doses of 0.01 mg/kg and 0.036 mg/kg of pyridostigmine (see Fig. 17). Occasional subjunctional mitochondria possessed circular regions of rarefied matrix. Other membrane-bound organelles were not noticeably affected. Sarcomeres were at rest length and the component myofibrils were unaltered in appearance. Inspection after continued drug exposure (up to 14 days) revealed similar alterations with no increase in frequency. Thus, prolonged, low-level ChE depression resulted in infrequent, subtle alterations of the matrix of a limited number of postsynaptic mitochondia.

High-dose subacute exposure (2-14 days) to pyridostigmine produced more remarkable postsynaptic changes. Swelling of

subjunctional membrane bound organelles was minimal to severe when present (Fig. 20), but was generally absent even following exposure to 20 mg of pyridostigmine over a 14 day period (Fig. 22). A second type of damage, disruption of myofibrillar components or abnormal contraction (shortening) of sarcomeres, was frequently observed in fibers (Figs. 21,22). Damage to membrane bound organelles and to myofibrils were not mutually dependent as they occurred separately, as well as, in the same fiber. When present, the sarcomere damage was limited to a small subjunctional region (Fig. 21). The extent of the myopathy with this type of drug exposure was similar to that observed in NMJs exposed to 0.36 mg/kg pyridostigmine by single subcutaneous injection. Both routes of administration decreased ChE activity to approximately 30%. Postsynaptic alterations did not increase in frequency or severity during the 14 day exposure period. It is important to note, however, that after 14 days of decreased ChE activity (30% of control), subjunctional sarcomeres frequently displayed unusual localized contraction (Fig. 22). In these cases, myofibrillar damage was not apparent. Essentially every fiber from animals in the high-dose minipump group possessed pyridostigmine-related alterations in the immediate subjunctional region. Many of the fibers appeared to be only minimally affected.

Recovery Following Pyridostigmine Exposure By Injection and Minipump. A 30 minute exposure of diaphragm (Fig. 23), soleus (Fig. 25) and EDL (Figs. 27 and 28) NMJs to 1.0 mg/kg of pyridostigmine resulted in ultrastructural changes very similar in nature to those observed after a 30 minute exposure to 0.36 mg/kg of drug. The only significant difference was a consistant tendency toward increased severity of the pathology. The most notable presynaptic alterations were evidenced as occasional aberrant mitochondria (Figs. 27,28), partial withdrawal of nerve terminal areas from postsynaptic junctional folds (Figs. 23, 28) or separation of the pre- and postsynaptic components by intervention of Schwann cell processes (Fig. 25, 28). Postsynaptic alterations were also similar to those observed at lower doses. Mitochondria frequently possessed rarefied or swollen regions (Figs. 23, 25, 28) and the myofibrillar organization of the sarcomeres was sometimes disrupted (Figs. 23, 28). While these various alterations appear differently in some of the micrographs of the three different muscles, it is important to note that considerable variation occurred. This variation is emphasized in Figs. 27 and 28 which reflect the difference in the extent of damage within the EDL. The damage to the NMJ in Fig. 28 appears modest in comparison to the partial denervation, as well as, mitochondrial and myofibrillar involvement apparent in Fig. 28. Similar disparity existed in the degree of damage evidenced from one NMJ to the next in the diaphragm and soleus muscles.

Similarly, a 14 day exposure to pyridostigmine via osmotic minipump (total exposure 20 mg) reflected the same types of alterations (Figs. 20, 23) observed with shorter drug exposure. However, partial denervation was more extensive in the NMJs of all three muscles and postsynaptic mitochondrial involvement was much less pronounced (Figs. 29, 31, 33).

Recovery was observed in all three muscles following both dosage regimens (Figs. 24, 26, 30, 32, 34) . The results of the acute studies are illustrated in the diaphragm (Fig. 23) and soleus (Fig. 26). Those of subacute exposure are illustrated in all three muscles (Figs. 30, 32, 33). While modest mitochondrial involvement sometimes persisted (Fig.26), this type of damage was not commonly observed (Figs. 23, 30, 32, 34). The most consistantly observed alteration remaining after 60 days of recovery from acute and subacute pyridostigmine exposure was Schwann cell intervention between the nerve terminal and junctional fold crests (Fig. 24, 32). In addition, postsynaptic nuclei often possessed very irregular margins and were frequently associated with numerous Golgi.

Following 90 days of recovery from acute and subacute pyridsotigmine exposure, myofibrillar structure and post-synaptic organelles appeared normal (Figs. 35, 35). Some nuclei exhibited highly irregular margins (Fig. 35). This state may be indicative of a highly active metabolic state.

Some localized separations of nerve terminal and junctional fold crests remained (Fig. 36). These occurrances were not quantified and thus may or may not be similar to control values for naturally occurring synaptic irregularities.

Technology for X-ray Microanalysis of Subjunctional Calcium Ions The x-ray microanalysisnstudies required the design and production of a specimen support. This was a time consuming trial-and-error process. The primary difficulty arose in establishing a design which would accommodate (1) the length of end-to-end muscle fibers and (2) at the same time localize and ensure optimum freezing of the neuromuscular junction region of the fibers. Figure 37 illustrates the final design. The conical shape of the specimen support permits a small bundle of muscle fibers to be mounted on the support with the neuromuscular junction band situated at the apex of the cone. This optimizes freezing in the desired area. The ends of the muscle fibers are tied with small lengths of suture and anchored by pins in small silastic-filled wells. This specimen support fits into the Gentlemen Jim freezing device which also had to be modified to hold the support for the freezing process. Due to the design, the apex of the cone (ie. the neuromuscular junction region) contacts the liquid nitrogen cooled copper block first and therefore optimizes freezing. The differences in quality of freezing in the junctional region and the remainder of the muscle fiber is apparent in Figure 38 which is a light level photomicrograph of a cross section through a frozen muscle bundle. Once frozen, samples can be (1) freeze-substituted, (2) stored on the specimen support in liquid nitrogen or (3) immediately sectioned. The specimen support also serves as a chuck for cyromicrotomy in the LKB Cryonova. Frozen sections can be transported from the microtome to the microscope using a Gatan Cyrotransfer device.

Contrast is minimal in transmission images of frozen sections, however, mitochondria and the sarcomere banding are generally recognizable (Figure 39). The few frozen and freeze substituted specimens which have been examined to date indicate a marked contrast in ion concentrations in junctional regions of muscle fibers from control (single subcutaneous injection of diluant) and pyridostigmine treated (single subcutaneous injection of 2.6 mg/kg pyridostigmine)animals. It appears that muscle fibers in the junctional regions of treated animals may have significantly altered calcium and phosphate levels. If this turns out to be confirmed following continued investigation of additional samples and with additional analytical techniques (ie mapping etc.), the data may form the basis for specific explanations of possible mechanisms of pyridostigmine induced morphological alterations.

For example, if the free calcium ion concentration in the cytosol is increased significantly above the very low normal concentration of (10^{-7}M, one could speculate that the major routes would be influx through open acetylcholine receptors (activated by excess acetylcholine in the cleft) and by efflux from the sarcoplasmic reticulum. Some of the free calcium would be expected to bind with troponin and initiate myofibrillar contraction which in turn would require hydrolisis of ATP. Under normal circumstances, a short period of contraction would normally be followed by release of calcium by

the troponin thus terminating contraction. This should be followed by reuptake of calcium by the sarcoplasmic reticulum, a step which also requires ATP. The mitochondria would also serve to sequester calcium if the cytosol underwent substantial increases in calcium concentration. The mitochondria use the electrochemical gradient across the inner mitochondrial membrane, generated during the electron transfer steps of oxidative phosphorylation, to drive the uptake of calcium from the cytosol. If, however, the calcium levels are significantly shifted toward levels present in the extracellular fluids (apx. 10^{-3}M) the normal cellular mechanisms for maintaining appropriate levels of calcium in the cytosol may not be able to cope. For example, if substantial concentrations of calcium are added to respiring mitochondria, the mitochondria cease ATP production completely, and all the energy in their electrochemical gradient is diverted to pumping calcium. Thus, the presence of pyridostigmine may accelerate the expenditure of ATP by sustained localized contraction and sarcomplasmic reticulum re-uptake of the ion and at the same time compromise the production of ATP by the mitochondria. It is more difficult to speculate on the effects of increased calcium on the production of ATP from phosphocreatine. However, it is possible that energy requirements which significantly exceed the norm would also diminish and/or deplete phosphocreatine resources.

DISCUSSION

Acute and subacute doses of pyridostigmine bromide which caused whole blood ChE depression of 60-90%, resulted in pre-and postsynaptic morphological alterations to every diaphragm, soleus and EDL NMJ analyzed. Presynaptic damage included swollen mitochondria and partial withdrawal of nerve terminal branches. These two alterations were sometimes observed in the same NMJ but also occurred independently. Mitochondrial changes presumably reflect altered ionic concentrations within the presynaptic compartment. Changes in the spatial relationship of the nerve terminal and the junctional fold crests were present as Schwann cell processes in the primary cleft and/or by withdrawal of the entire terminal portions. Schwann cell processes have been reported as a regular feature in the primary clefts of frog NMJs, but occur only rarely in the control NMJs of rat muscles. Regional withdrawal of a small proportion of mammalian NMJs occurs on a continuing basis in the normal process of synaptic turnover (Cotman, et al., 1981). In the case of pyridostigmine-treated NMJs, Schwann cell processes in the cleft and partial withdrawal of nerve terminals act as a type of partial denervation. Both phenomena effectively reduce the amount of optimally functioning synaptic surface available. It is apparent that this decrease in NMJ functional area is enhanced by pyridostigmine exposure although the mechanism responsible for the change is not known. It should be noted that the reduction in apposing pre-and postsynaptic surfaces does not necessarily imply a concomitant reduction in neuromuscular function. Some portions of each NMJ appear morphologically sound and may be able to function at a level which does not significantly impair animal behavior. Appropriate electrophysiological studies are required to assess the effect of decreased synaptic area on function.

Pyridostigmine-induced postsynaptic damage included changes in membrane bound organelles, most notably the mitochondria, and/or disruption of sarcomere organization. These two categories of alterations sometimes occurred in the same NMJ but were not mutually dependent. Abnormalities in membrane bound organelles presumably reflect inappropriate ion concentrations resulting from drug exposure. Extremely high levels of Ca accumulate in the endplate regions (Salpeter, et al., 1982). These high levels of Ca may also result in disruption of myofibrillar integrity by mediating Ca -activated proteases which attack particular muscle proteins (Salpeter, et al., 1982). The myofibrillar changes associated with the acute and subacute doses of pyridostigmine used in this investigation are mild in comparison to those seen at higher acute doses (Hudson, et al., 1985).

Since the diaphragm, soleus and EDL have different fiber

type compositions (Hudson, et al., 1982), the possibility existed that exposure to pyridostigmine might affect the three muscles differently. Similarly, drug administration by single injection resulted in a rapid rate of ChE inhibition, with maximum ChE depression occurring approximately 30 minutes postinjection, while a minipump inhibited ChE more slowly, with maximum ChE depression occurring 4-12 hours following the minipump implant. Thus, it was further possibile that the mode of drug administration might result in differences in the severity of morphological alteration. These data indicate that acute and subacute doses of pyrodistigmine which produced an approximate ChE depression of 60-70% resulted in morphological alterations to diaphragm, boleus and EDL NMJs which were variable within each muscle analyzed. The diaphragm appeared to exhibit greater pre- and postsynaptic alterations in at least some NMJs. However, the differences were not significant or consistant enough to allow a subjective determination that the diaphragm is more sensitive to pyridostigmine exposure. Quantitation of the alterations would be necessary to determine positively whether significant differences exist in the drug sensitivity levels of the three muscles. Thus, at these dose levels, drug-induced alterations did not appear to be selectively associated with a specific muscle type or mode of drug administration after analysis of animals subjected to short-term exposure. It is possibile that selectivity might be observed at different dose levels.

With increased periods of analysis following single injection or 14 osmotic minipump exposure, recovery was evident in all three muscles. While the evidence is somewhat subjective, it appears that the nuclei in NMJs from animals in the 14 day subacute exposure group possessed aberrant nuclei with extremely irregular margins after 60 days of recovery than did the NMJs from animals in the acute exposure group. The significance of this observation is not currently known. However, nuclei of this type are normally associated with cells with very high metabolic levels.

The following conclusions were drawn:

1. At a ChE depression of 60-70 %, no discernible difference in the severity of NMJ damage is apparent between pyridostigmine exposures by single injection and by osmotic minipump.

2. Ultrastructural alterations to NMJs of diaphragm, soleus and EDL were similar in extent and nature following short-term exposure to pyridostigmine. Thus, at a ChE depression of 60-70 %, pyridostigmine exposure does not appear to selectively affect one muscle fiber type more severely than another.

3. Diaphragm, soleus and EDL NMJs all reflect evidence

of recovery from acute and subacute exposure to pyridostigmine following a 90 day drug-free period. Current data indicate that the soleus may require a more extensive recovery period than the diaphragm and EDL.

RECOMMENDATIONS

Pyridostigmine-induced alterations occurred at the NMJs of the three muscle types studied and thus established the toxic effects of the drug on fast twitch (EDL), slow twitch (soleus) and mixed (diaphragm) muscles. However, strong evidence that the damage which follows pyridostigmine exposure is reversible in all three muscles. Furthermore, recovery processes were evidenced even after moderately high drug exposures which induced whole blood ChE depressions of 70-90%. Since the potential for recovery from pyridostigmine-induced damage appears to be substantial, further evaluation of the drug as a potential prophylactic agent against intoxication by organophosphate agents is strongly encouraged.

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Figure 1. The percent whole blood ChE activity present 30 minutes after a single injection of pyridostigmine was not significantly altered at doses of 0.0036 or 0.006 mg/kg drug. However, only 70% ChE activity remained 30 minutes after a single injection of 0.01 mg/kg pyridostigmine. A dose related decrease of ChE activity was observed with doses greater than 0.006 mg/kg of pyridostigmine. (Minimum n=4; maximum n=16)

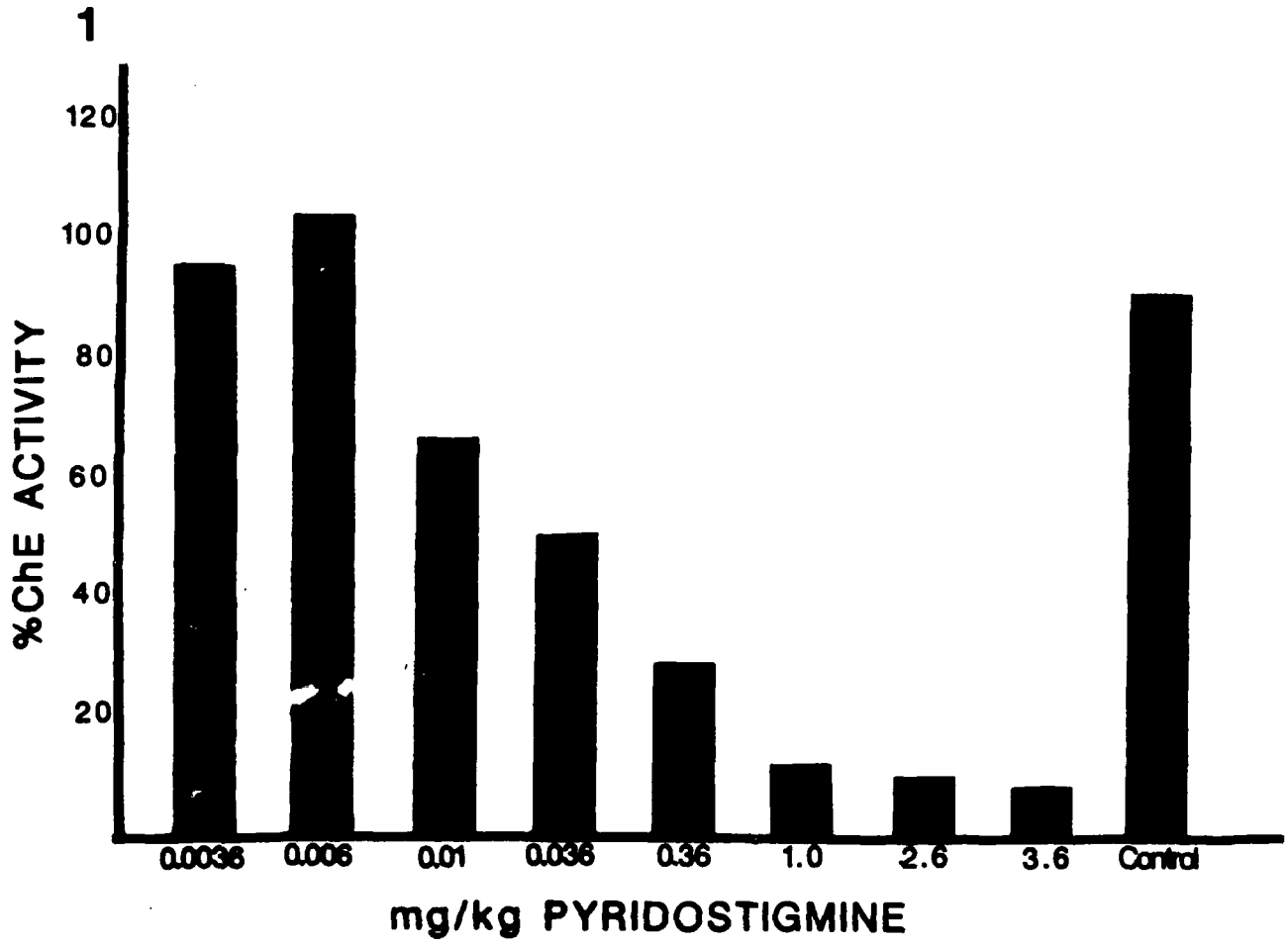

1
120
100
80
60
40
20
%ChE ACTIVITY
0.0036
0.006
0.01
0.036
0.36
1.0
2.6
3.6
Control
mg/kg PYRIDOSTIGMINE

Figure 2. The percent whole blood ChE activity was depressed by continuous infusion of 1.5 mg/ml and 10.0 mg/ml pyridostigmine via 2 ml osmotic minipumps. The percent decrease in ChE activity was related to the concentration of drug in the minipuimp but did not increase with cumulative dose. (minimun n=4; maximum n=18)

2

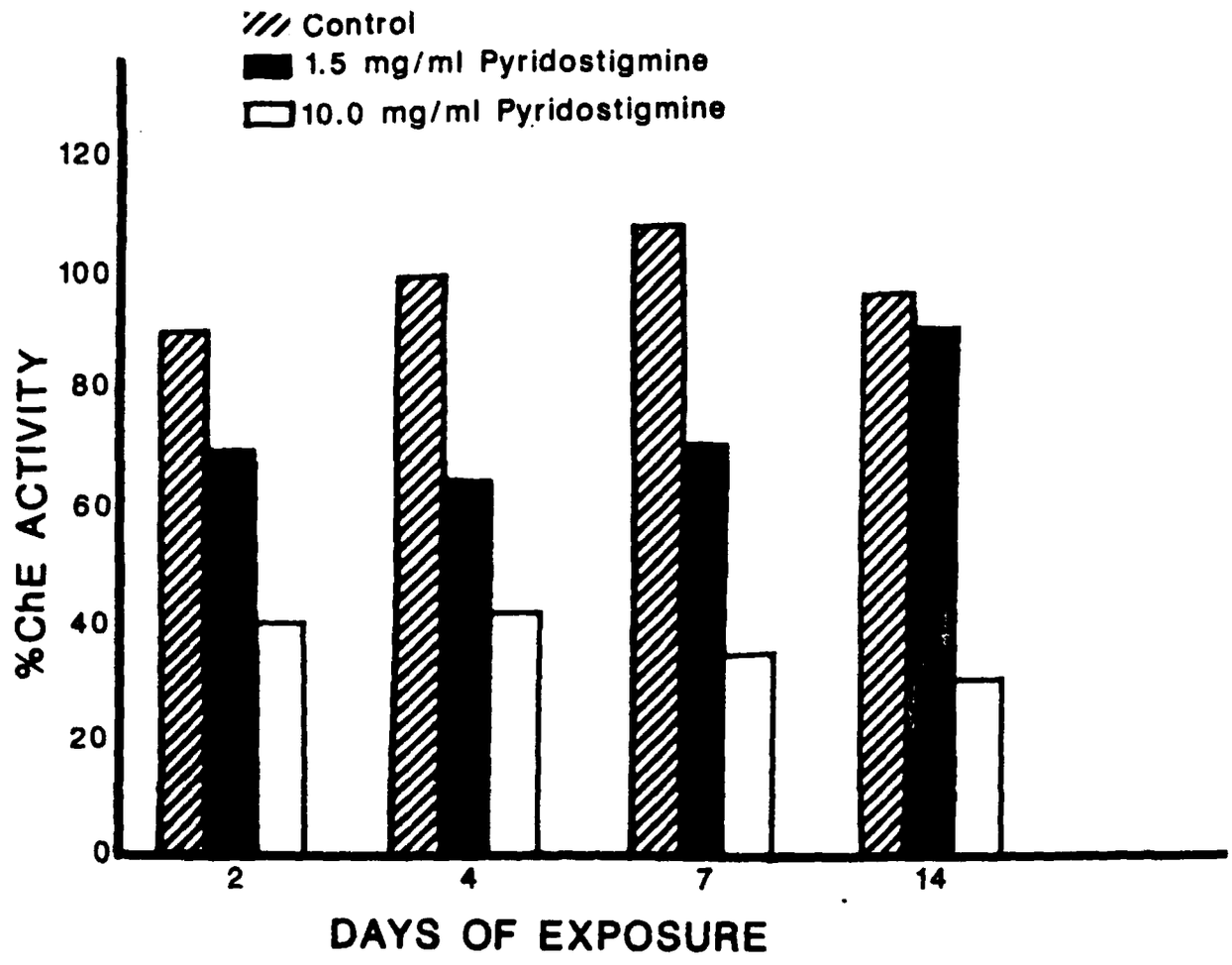

Control
1.5 mg/ml Pyridostigmine
10.0 mg/ml Pyridostigmine
%ChE ACTIVITY
120
100
80
60
40
20
0
2
4
7
14
DAYS OF EXPOSURE

Figure 3. The percent whole blood ChE activiaty was monitored at intervals up to 90 days following single injections of 0.036 mg/kg pyridostigmine and 1.0 mg/kg pyridostigmine. Recovery of ChE activity was rapid following both doses and remained within control levels through the 90 day recovery period.

3

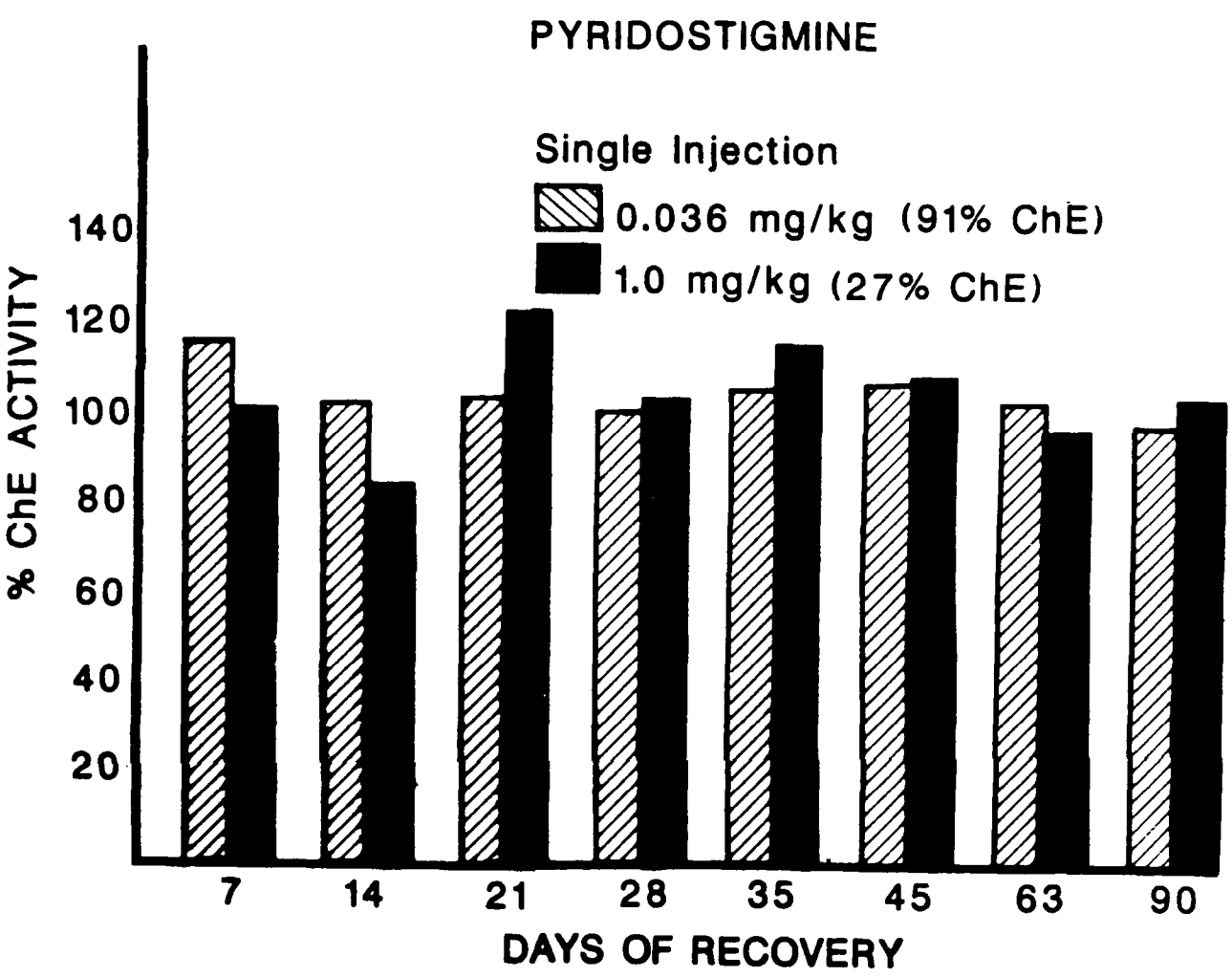
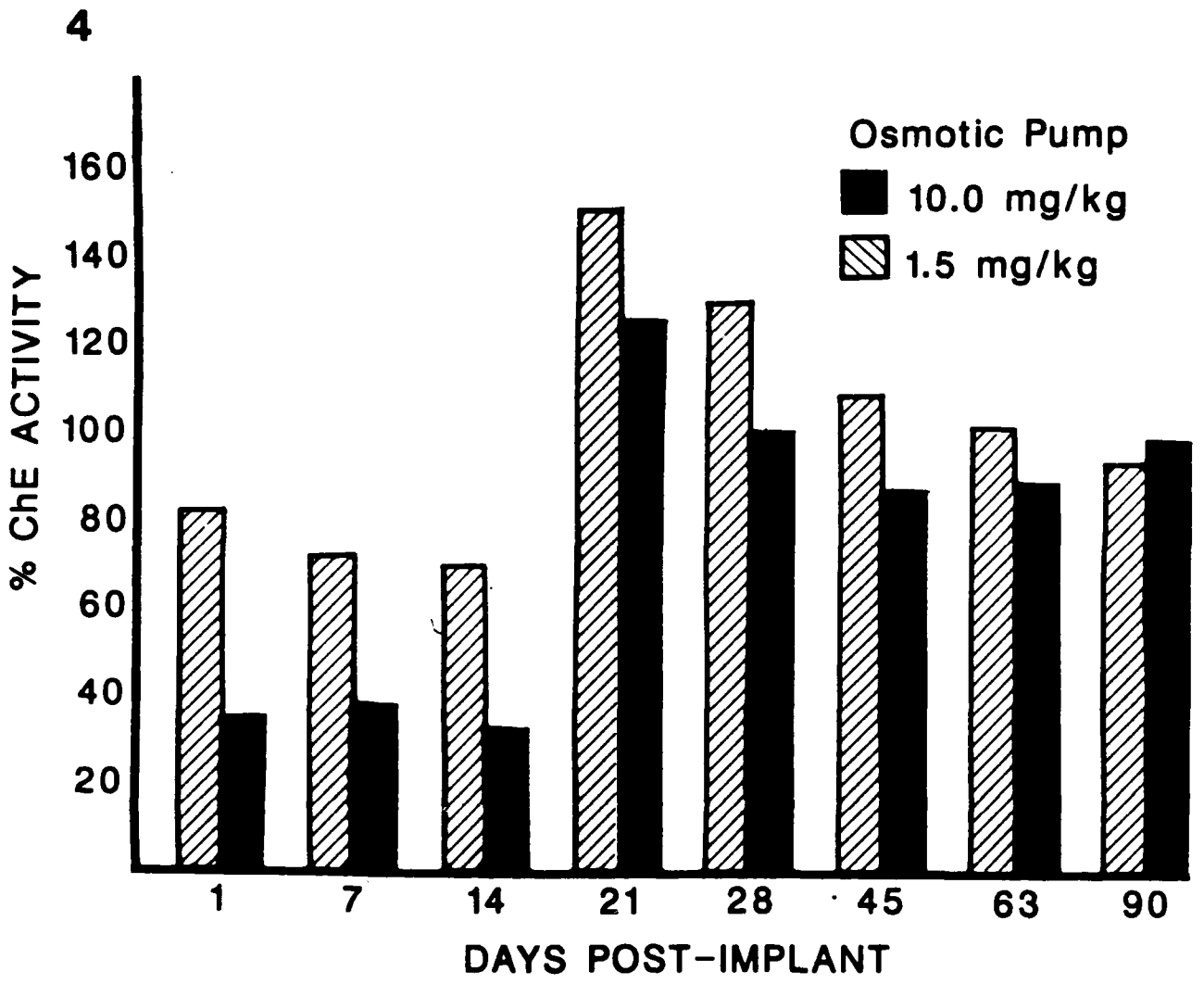

PYRIDOSTIGMINE
Single Injection
0.036 mg/kg (91% ChE)
1.0 mg/kg (27% ChE)
% ChE ACTIVITY
140
120
100
80
60
40
20
7
14
21
28
35
45
63
90
DAYS OF RECOVERY

Figure 4. The percent whole blood ChE activity was monitored at intervals up to 90 days following continuous infusion of 1.5 mg/ml and 10.0 lmg/ml pyridostigmine via 2 ml osmotic minipumps. Recovery from drug-induced decrease in ChE activity was rapid following both doses.


4
Osmotic Pump
10.0 mg/kg
1.5 mg/kg
% ChE ACTIVITY
160
140
120
100
80
60
40
20
1
7
14
21
28
45
63
90
DAYS POST-IMPLANT

Figures 5,6,7. Electron micrographs of neuromuscular junctions from control diaphragms. Fig. 5 illustrates the typical structural features of an untreated control NMJ. Fig. 6 illustrates a NMJ from a control rat exposed to a single injection of Mestinon-equivalent buffer 30 minutes prior to fixation, and Fig. 7 is a typical NMJ from a control rat subacutely exposed to Mestinon-equivalent buffer for 14 days prior to fixation. All three synapses reveal the typical close apposition of the nerve terminal (nt) to the junctional folds (jf) with basal lamina evenly interposed in the primary cleft. Numerous mitochondria (m) are present. The dense Z-line and I, A and H bands are readily identifiable in the rest length sarcomeres. The muscle cell nucleus (Nu, Fig. 6) is typical of those observed in all three types of control preparations.

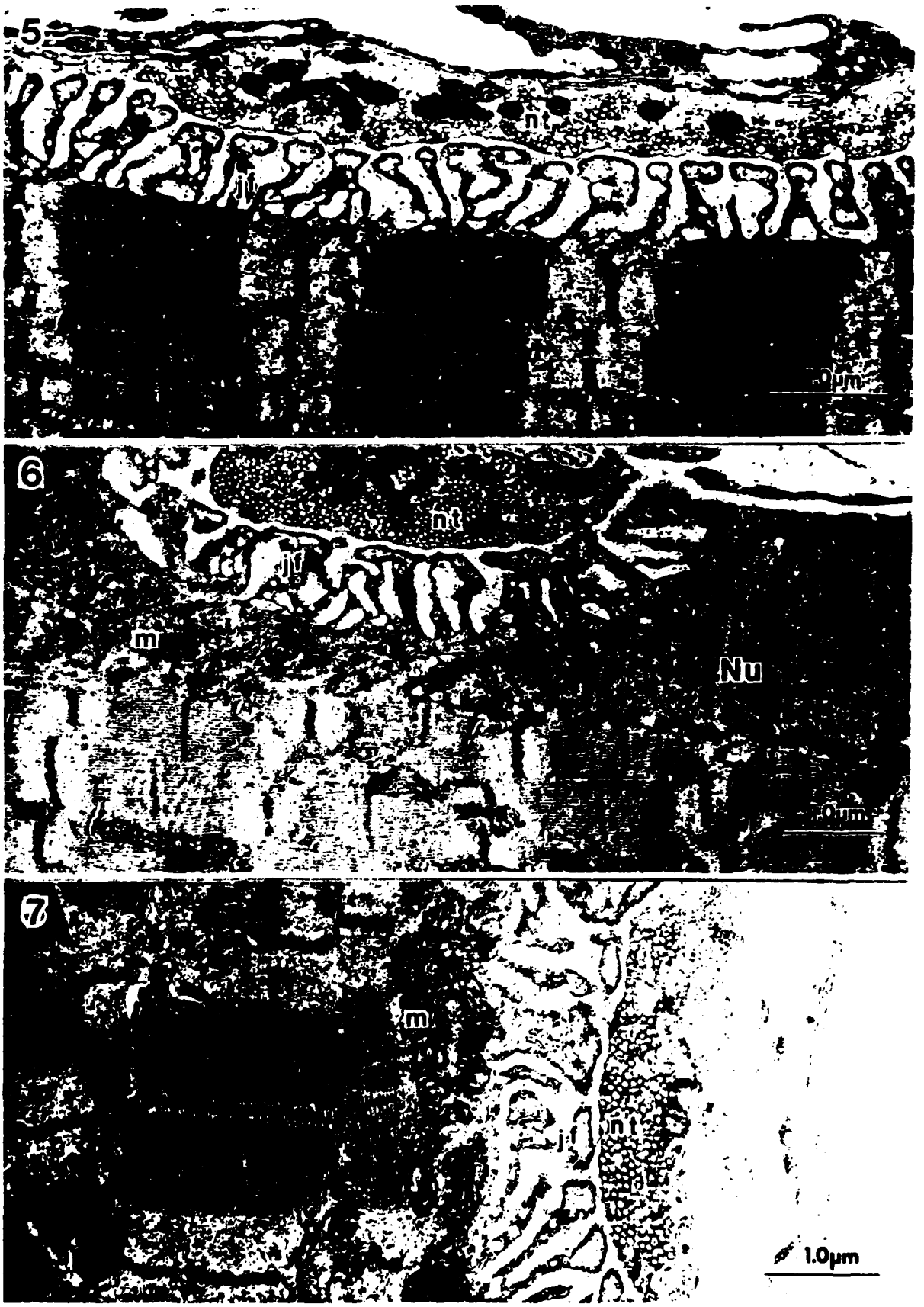

5
nt
6
nt
m
Nu
7
m
nt
j
1.0μm

Figure 8. With fixation 30 minutes after a single injection of 0.036 mg/kg of pyridostigmine the junctional folds (jf) and myofibrillar apparatus (mf) are normal, but the presynaptic ultrastructure is altered. The nerve terminal (nt) is normally apposed to the junctional folds, but damage appears in the form of multiple membranous layers (arrow) some of which surround vesicle-containing nerve terminal portions (see right of arrow). Vesicles, abnormally large in diameter, are evident in the nerve terminal. The arrowhead points to a Schwann cell (s) process that has invaded the nerve terminal from the nonsynaptic side.

Figure 9. Following a single injection of 0.36 mg/kg of pyridostigmine, all compartments in this neuromuscular junction are obviously affected. The nerve terminal (nt) contains altered mitochondria (m) and abnormal vesicular inclusions. The nerve terminal is withdrawn from various regions (asterisks) of the junctional folds while in other areas normal synaptice morphology is preserved. Vesicular debris, Schwann cell processes, and small nerve terminal profiles are evident in the regions of withdrawal.


8
scn
nt
mf
1.0μm

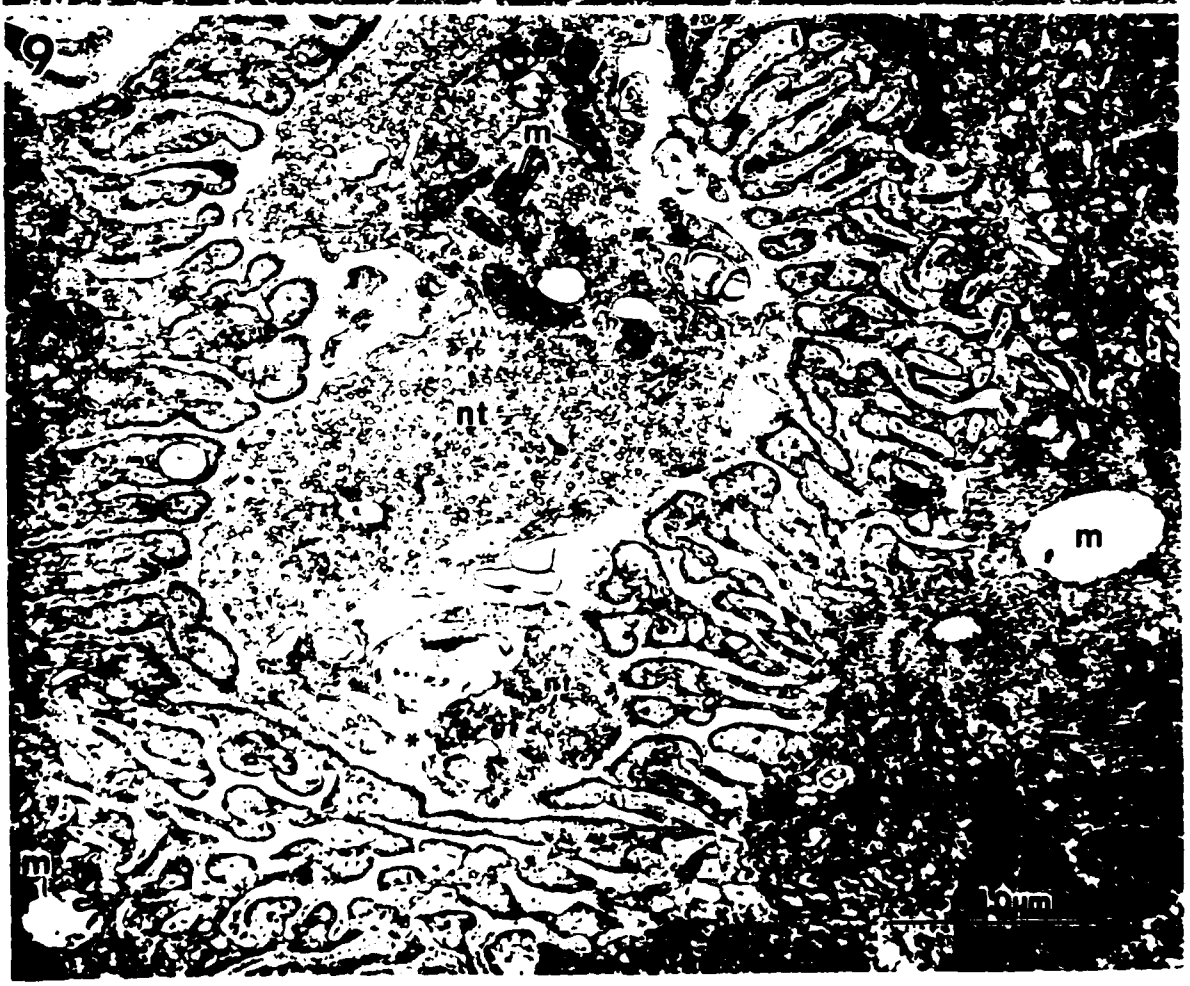
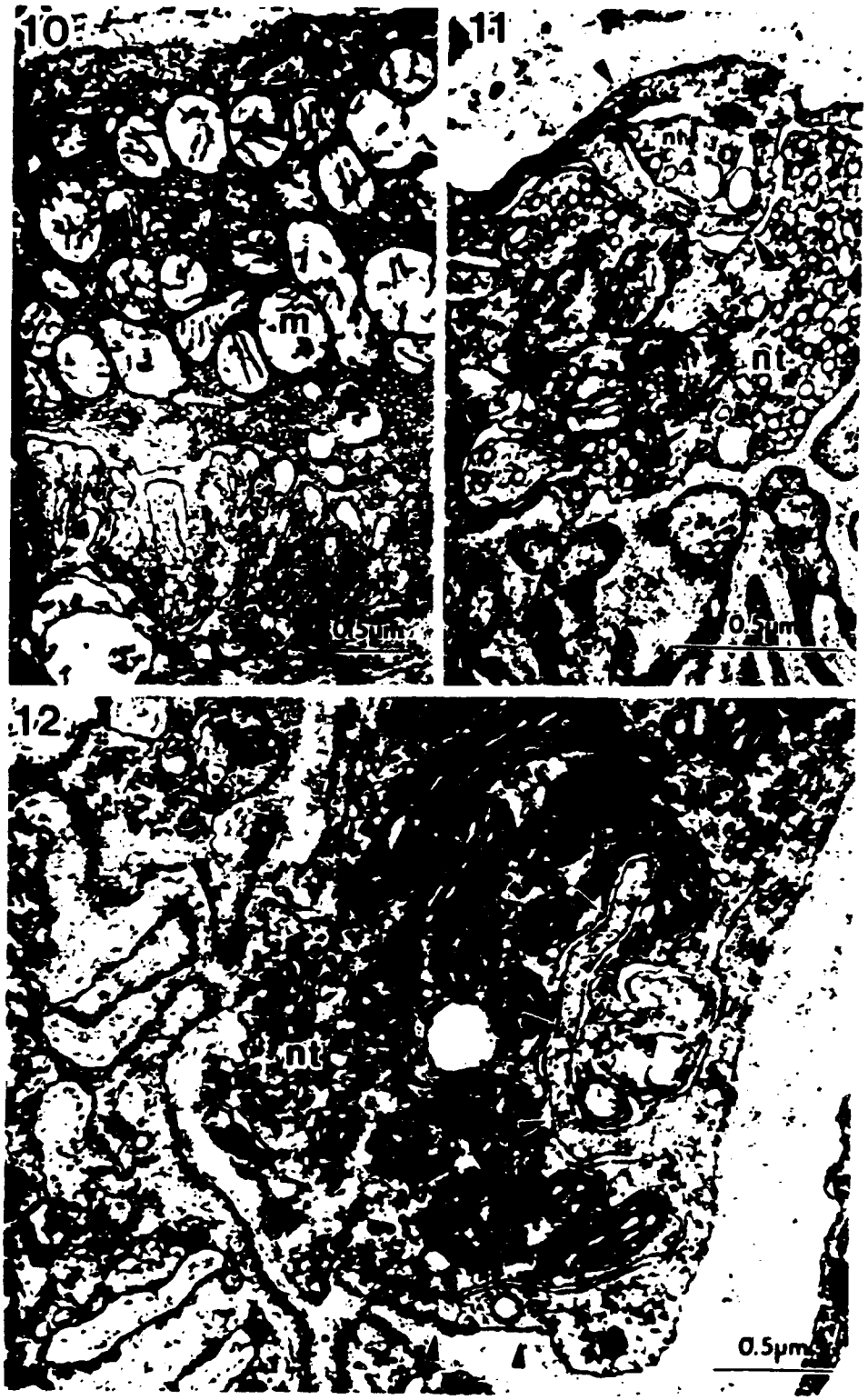

9
m
nt
m
mf
1.0μm

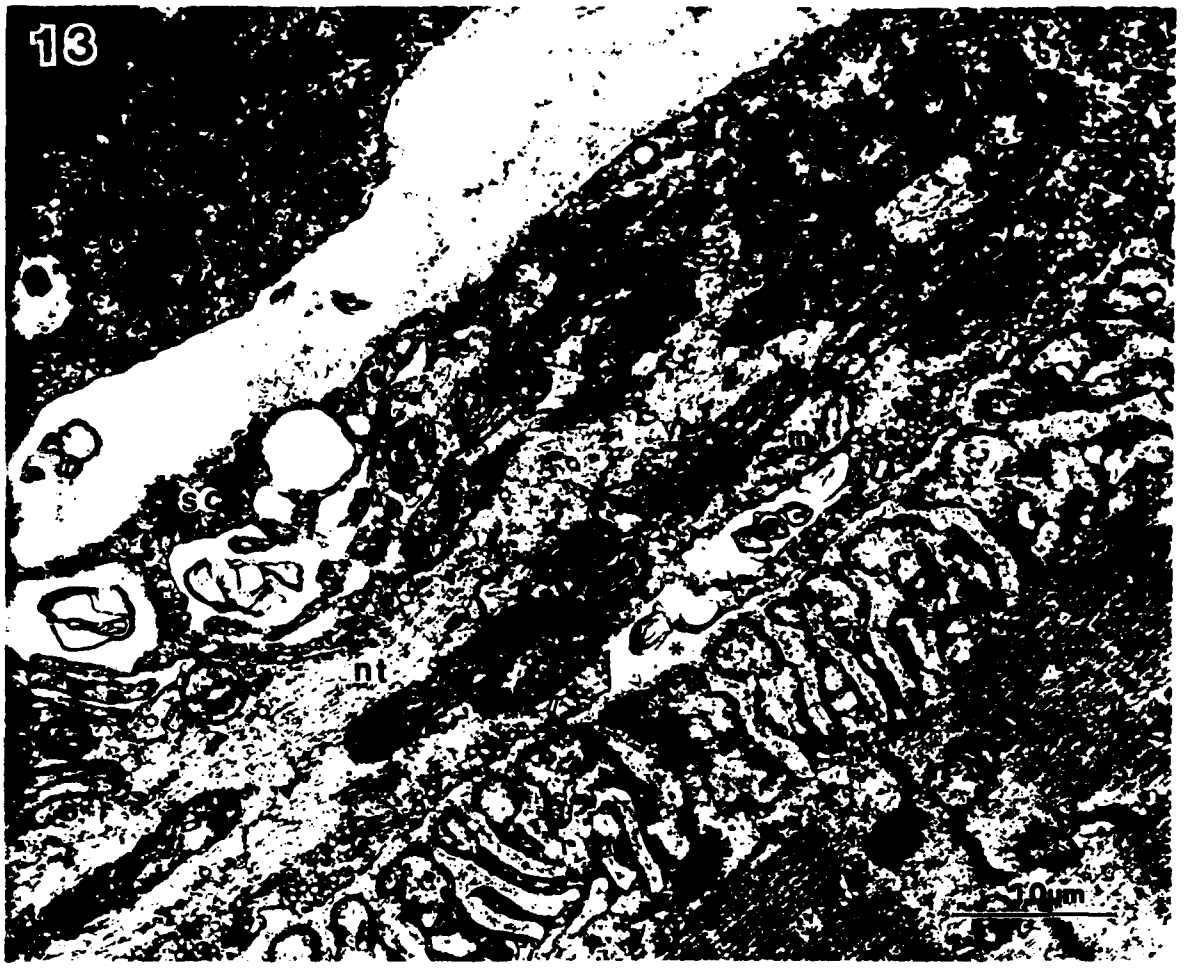
Figure 10. A single injection of 2.6 mg/kg of pyridostigmine resulted in altered presynaptic mitochondria (m) and a Schwann cell process (asterisk) separating the pre- and postsynaptic membranes.

Figure 11. Nerve terminals from pyridostigmine-exposed animals (0.036 mg/kg pyridostigmine, 30 min) were invaded by Schwann cell processes (arrowheads). In this plane of section, the vesicle-containing profile appears to be sequestered by Schwann cell processes. This is probably a plane of section artifact (e.g. dotted line in Fig. 12).

Figure 12. Fixation 17 minutes following a single injection of pyridostigmine illustrates a) that presynaptic alterations occur relatively rapidly after drug administration; b) that there is not always more damage with a higher dose; and c) that the separation of portions of nerve terminal is by Schwann cell projections a consistent feature of the drug-induced alterations. In this electron micrograph, a Schwann cell process has invaded the primary synaptic cleft (two arrowheads at bottom). The nerve terminal (nt) contains abnormal vesicular profiles and has withdrawn from the junctional folds (asterisk). The nonsynaptic side of the nerve terminal has been invaded by a Schwann cell process (three arrowhead). The dotted line indicates a plane of section that would yield the inappropriate impression that a portion of a nerve terminal had been sequestered within a Schwann cell.


10
m
0.5μm
11
nt
0.5μm
12
nt
0.5μm

Figures 13 and 14. A 2 day minipump exposure to pyridostigmine (1.5 mg/ml; 0.43 mg total dose) resulted in alterations. In Figure 11, note the difference in homogeneity of matrices of the various mitochondria (m). Regional withdrawal (asterisk) of the nerve terminal (nt) from the junctional folds is apparent in both figures although it varies greatly in extent. The NMJ in Figure 12 represents the most extensive separation of pre- and postsynaptic elements observed at this exposure. Schwann cell processes (s) are sometimes present in the primary cleft (arrowhead, Fig. 14).


13
SC
nt
0.1μm

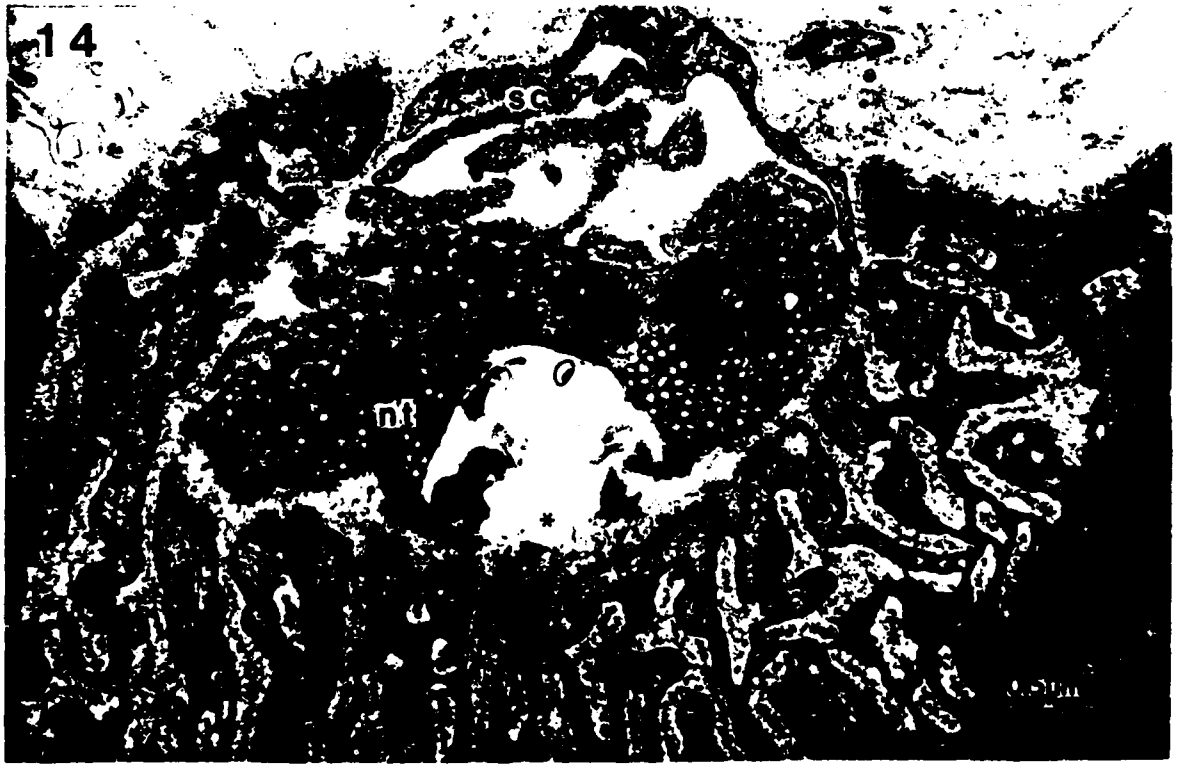

14
SC
nt

Figure 15. A 7 day minipump exposure to pyridostigmine (10 mg/ml; 10 mg total dose) resulted in both pre- and postsynaptic alterations. A portion of the NMJ (between arrows) is devoid of nerve terminal, while Schwann cell processes (arrowheads) have invaded another portion. The nerve terminal (nt) has some abnormal mitochondria.

Figure 16. A 14 day minipump exposure to pyridostigmine (10 mg/ml; 20 mg total dose) resulted in significant presynaptic alterations. Schwann cell (s) fingers (arrowheads) have invaded the primary cleft and the nerve terminal has withdrawn (asterisks) from the junctional folds (jf). Some nerve terminal mitochondrdia (m) are abnormal.

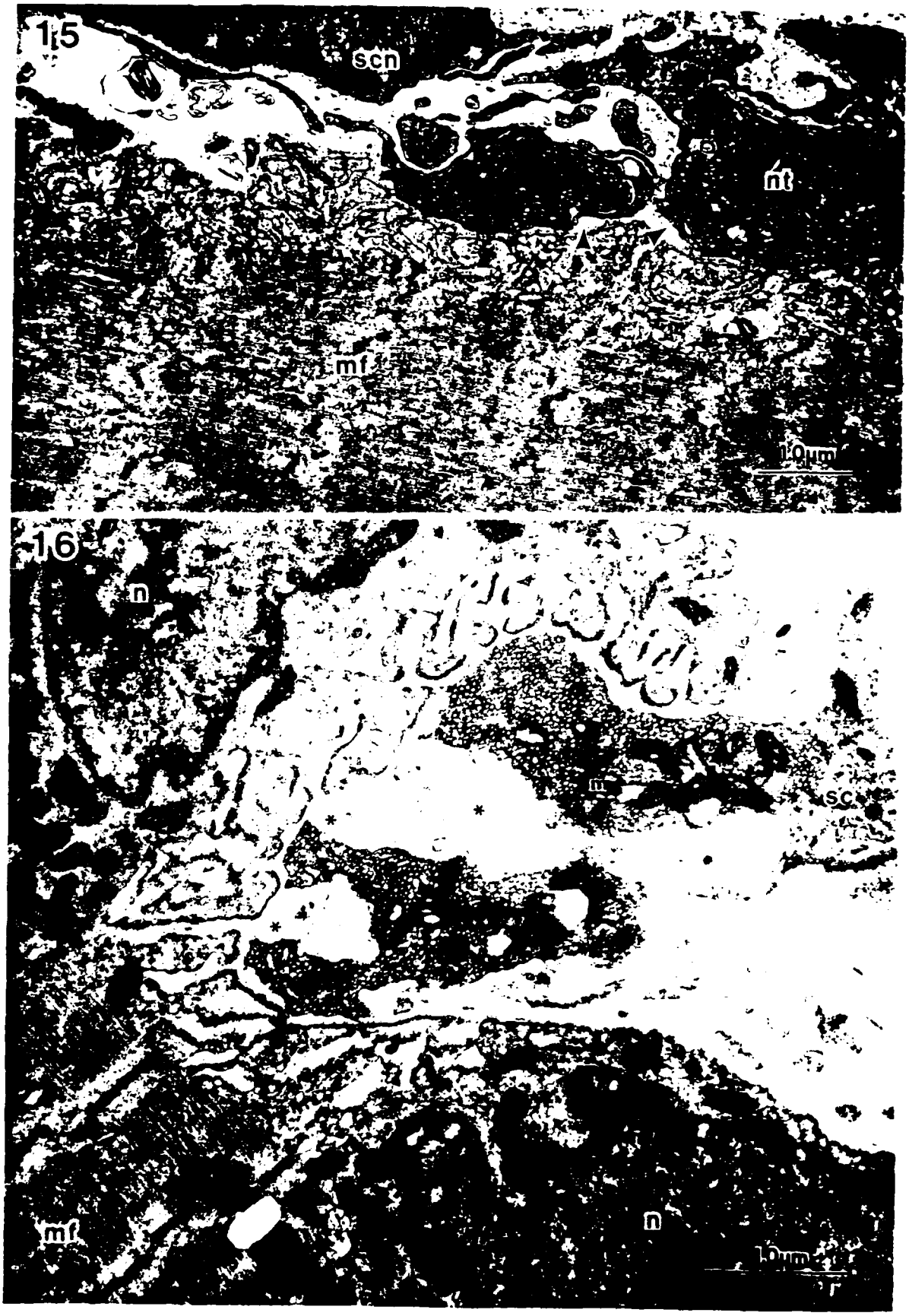

1.5
scn
nt
mf
1.0μm
1.6
n
m
sc
mf
n
1.0μm

Figure 17. A NMJ exposed to 0.036 mg/kg pyridostigmine exhibits normal junctional folds (jf) and myofibrillar apparatus (mf), the presynaptic ultrastructure is altered. The nerve terminal (nt) is normally apposed to the junctional folds over much of this field. However, damage appears in the form of multiple membranous layers (arrow) some of which surround vesicle-containing nerve terminal portions. Vesicles, abnormally large in diameter, are evident in the nerve terminal. The arrowhead points to a Schwann cell (s) process that has invaded the nerve terminal from the nonsynaptic side.


517
1.0μm

Figure 18. Following exposure to 0.36 mg/kg pyridostigmine, the nerve terminal of this NMJ contains altered mitochondria (m) and abnormal vesicular inclusions. The nerve terminal (nt) is withdrawn from various regions (asterisks) of the junctional folds while in other areas normal synaptic morphology is preserved. Vesicular debris, Schwann cell processes, and small nerve terminal profiles are evident in the regions of withdrawal.

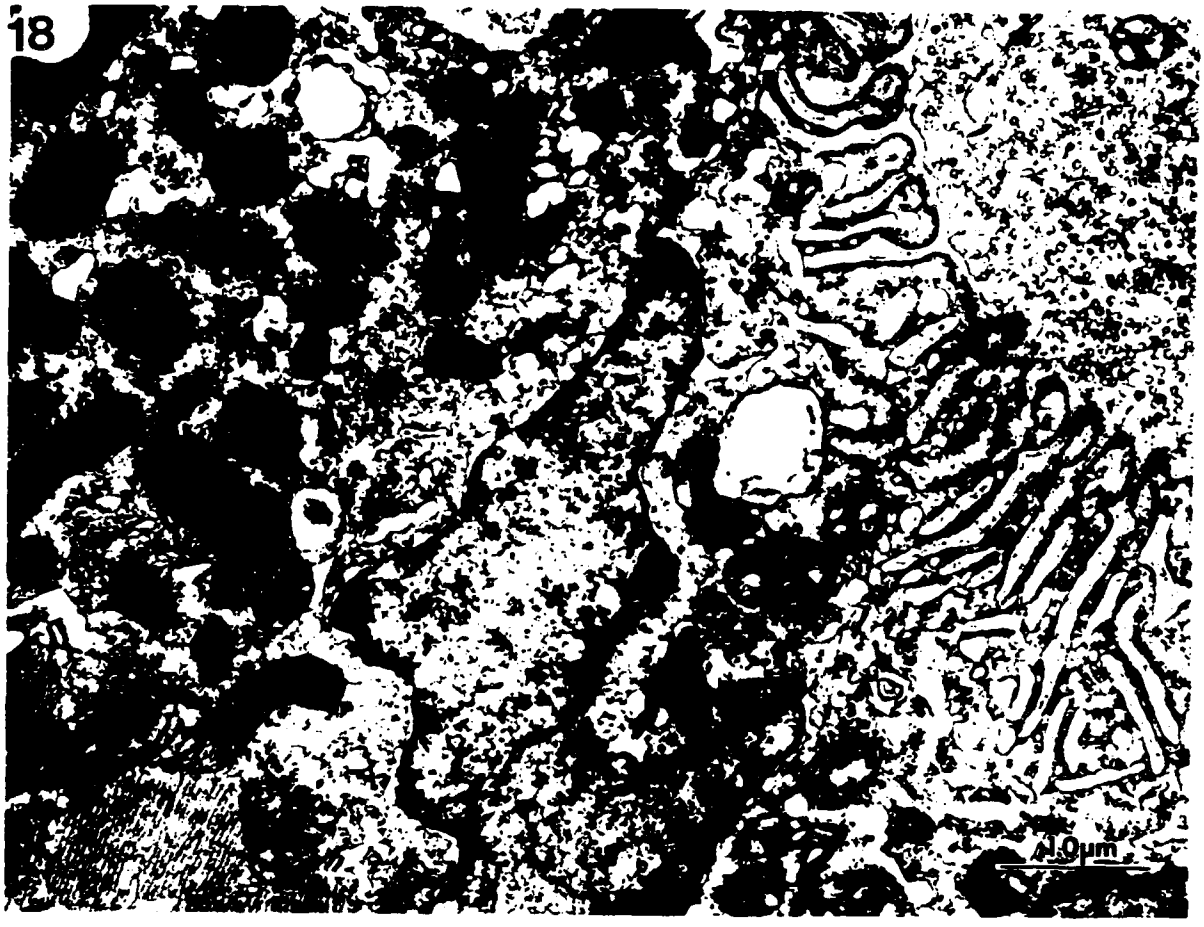

18
1.0μm

Figure 19. A single subcutaneous injection of 2.6 mg/kg pyridostigmine frequently results in supercontraction (between arrows) of the subjunctional sarcomeres with attendant gross mitochondrial (m) and myofibrillar damage. A distinct sarcomere banding pattern is not recognizable. Noncontractile muscle cell organelles including the mitochondria and nuclei (Nu) and the adjacent nerve terminal (nt) have been pushed out of normal position by supercontraction of the underlying myofibrils. The myofibrillar and mitochondrial alterations are graded with distance from the junction.


19
DT

Figures 20, 21. Micrographs of NMJs prepared after a 2 day exposure to pyridostigmine via high dose osmotic pump (2.8 mg total exposure) contrast the variability of drug effect on muscle fibers from the same animal. Figure 20 reflects noticeable alterations in postsynaptic mitochondria. Some have circular rarefied regions (arrowheads) while more severely affected mitochondria (m) have gross swelling of the inner compartment. The myofibrillar components appear minimally altered. In Figure 21, some llamellar structures (asterisk) are present. It is not clear if these are mitochondrial in origin. No marked swelling of mitochondria (m) or nuclei(Nu) is apparent. However, a number of sarcomeres lack Z-lines and are represented only by the presence of unorganized myofibrils (arrowheads). Note the normal spatial relationship of the nerve terminal (nt) and junctional folds (jf).


20
10μm

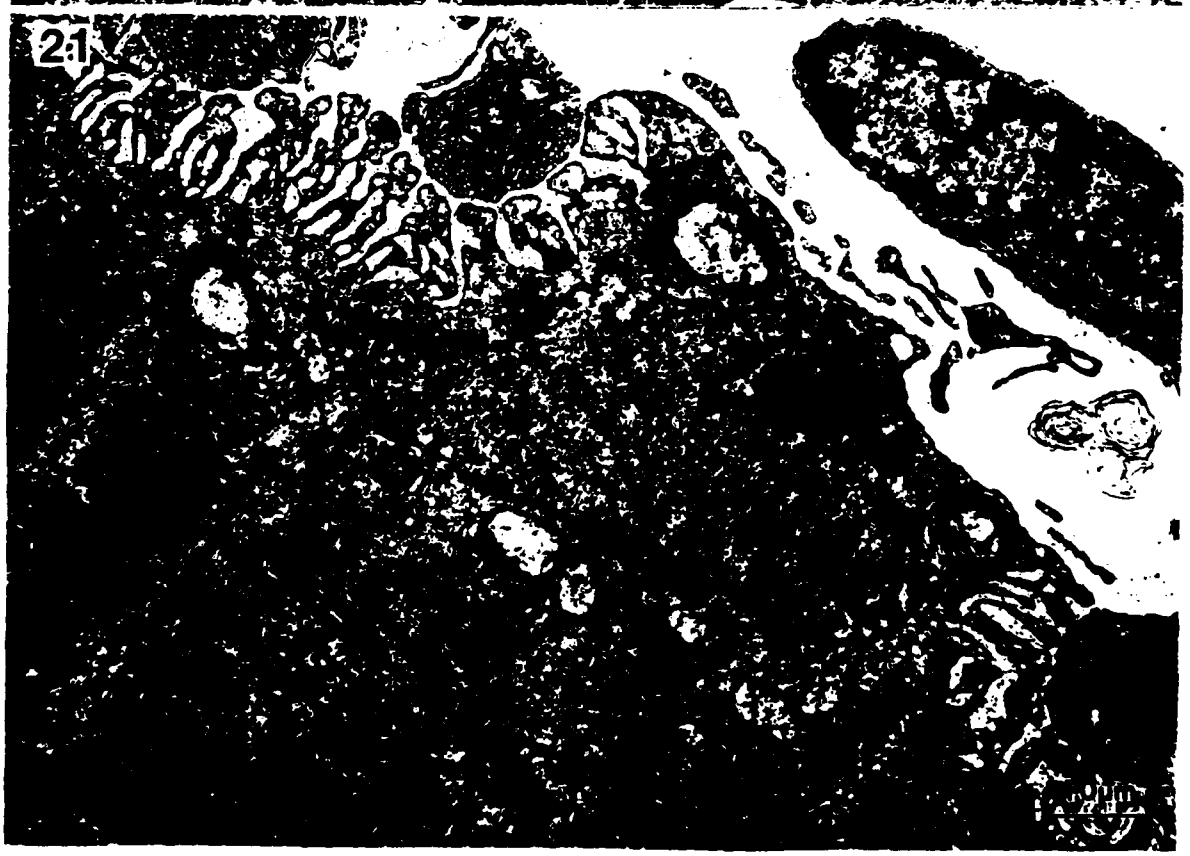
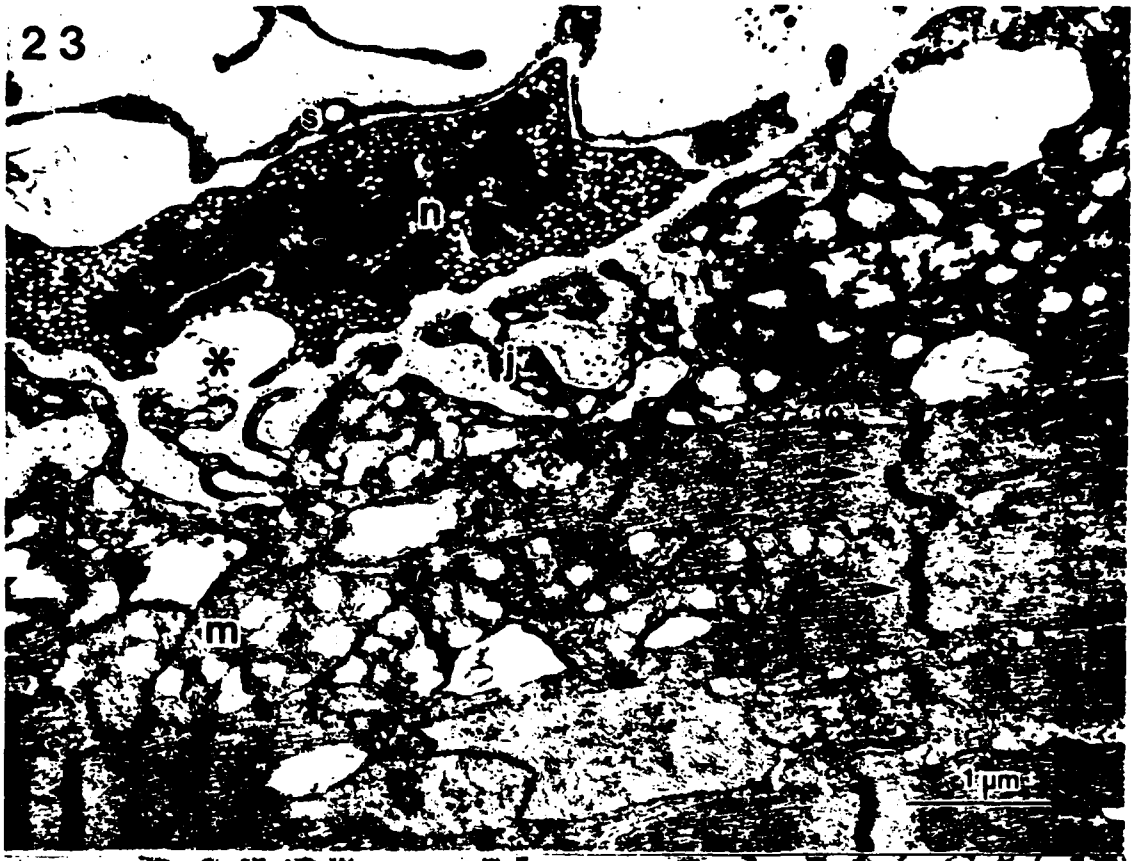

21

Figure 22. Following a 14 day high dose osmotic pump exposure to pyridostigmine (20 mg total exposure), the subjunctional mitochondria (m), nuclei (Nu), and endoplasmic reticulum of this NMJ appear morphologically unaltered. The Z-lines and A and I bands are clearly discernible. However, the sarcomeres are contracted to approximately 65% of rest length making the H-band indistinguishable. Compare the length of sarcomeres a and b with nonjunctional sarcomere c in the adjacent fiber.

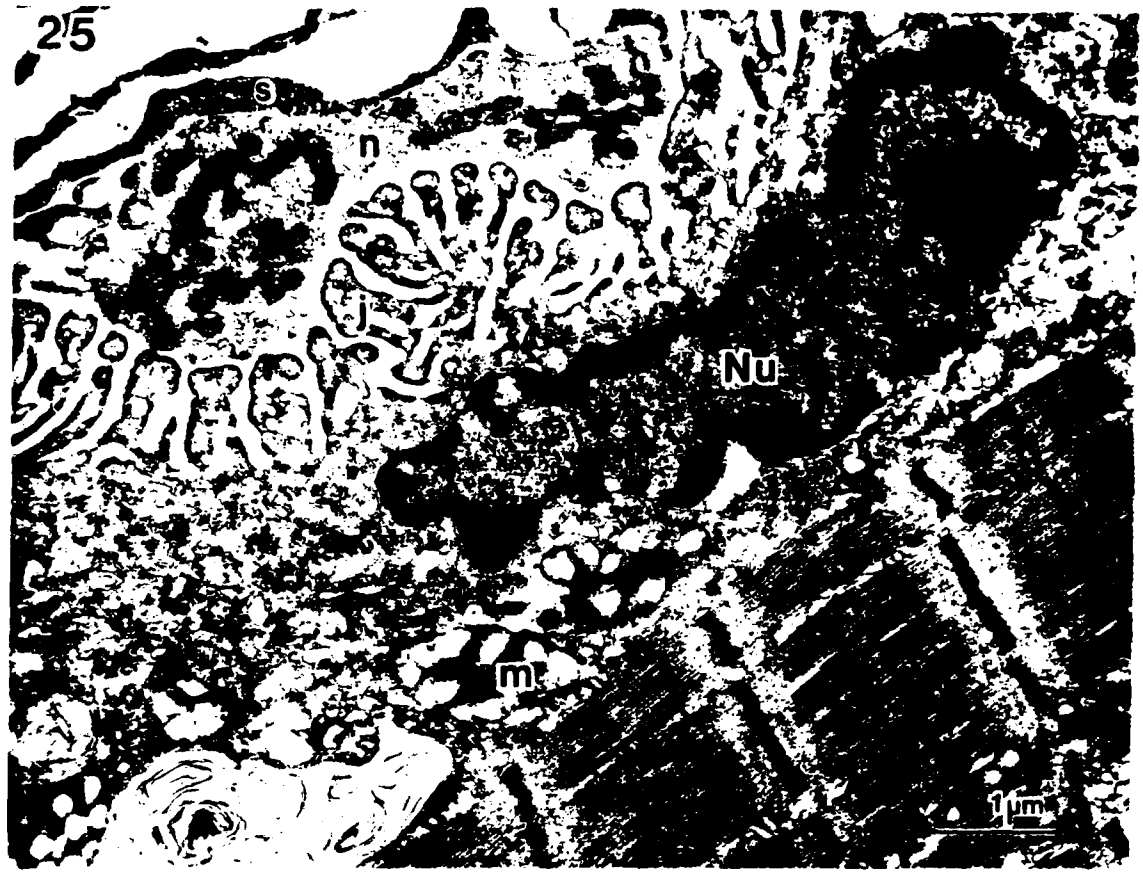

22

Figures 23 and 24. These diaphragm NMJs are from rats exposed to single injections of 1.0 mg/kg of pyridostigmine. The NMJ in Fig. 23 was fixed 30 minutes postinjection while the NMJ in Fig. 24 was fixed 60 days postinjection. The 30 minute exposure has induced regional separation of the nerve terminal from the junctional fold crests (asterisk) and large rarefied areas in the subjunctional mitochondria. In addition, some Z bands and myofibrils have lost the precise organization normally present. Sixty days following exposure, mitochondrial and sarcomere damage are no longer present. However, regional separation (*) of pre- and postsynaptic elements by Schwann cell (s) intervention in the cleft is common in this NMJ.


23
s
n
*
m
1 µm

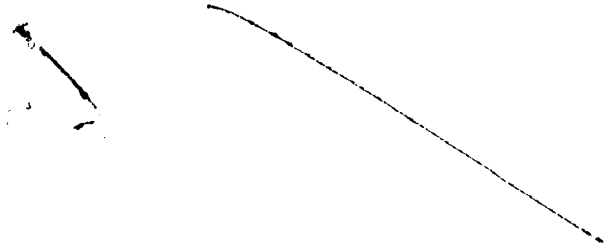

24
s
n
*
s
*
m
1µm

Figure 25 and 26. NMJs from soleus muscles exposed to 1 mg/kg. The muscles were collected at 30 minutes (25) and 60 days (26) postinjection. The nerve terminal shows no marked signs of alteration. However, a small process of Schwann cell is present in the synaptic cleft (arrow). With this short period of exposure, the postsynaptic mitochondria (m) are obviously affected. A large area containing multi-layered membrane fragments is apparent. Even after a recoveryk period of 60 days, damage persists in the second NMJ. Some presynaptic mitochondrial damage is evident. Postsynaptically, multilamellar bodies (arrows) persist.

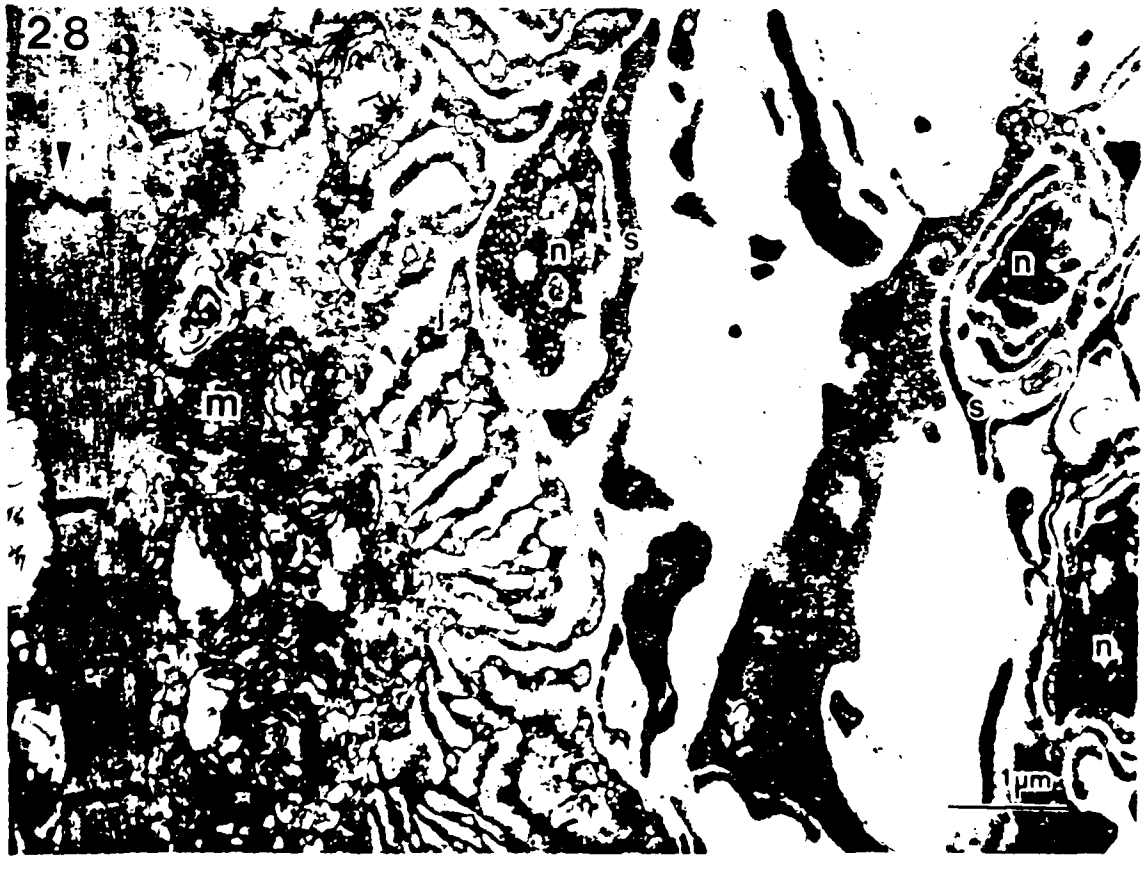

25
s
n
Nu
m
1μm


26
n
m
1μm

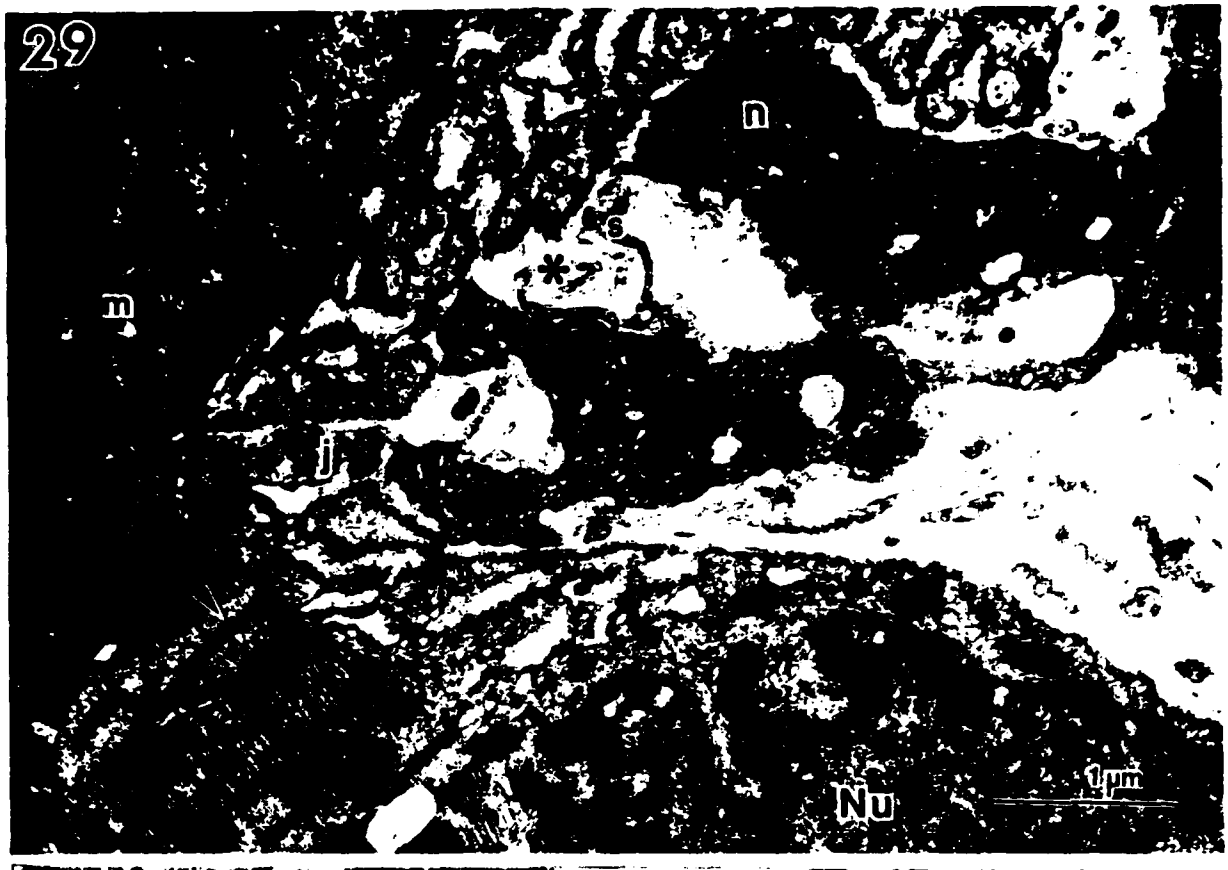
Figures 27 and 28. These two EDL NMJs illustrate the variation in the extent of damage in different muscle fibers 30 minutes after a single injection of 1.0 mg/kg of pyridostigmine. The NMJ in Fig. 27 possesses moderate alterations in presynaptic mitochondria and has apparent residual bodies present postsynaptically. The NMJ in Fig. 28 exhibits considerable ongoing changes in ultrastructure. Portions of nerve terminal are surrounded by Schwann cell in the right-hand area of the micrograph reflecting probable partial denervation processes. Postsynaptically, the mitochondria (m) are swollen and the myofibrillar components lack typical organization.

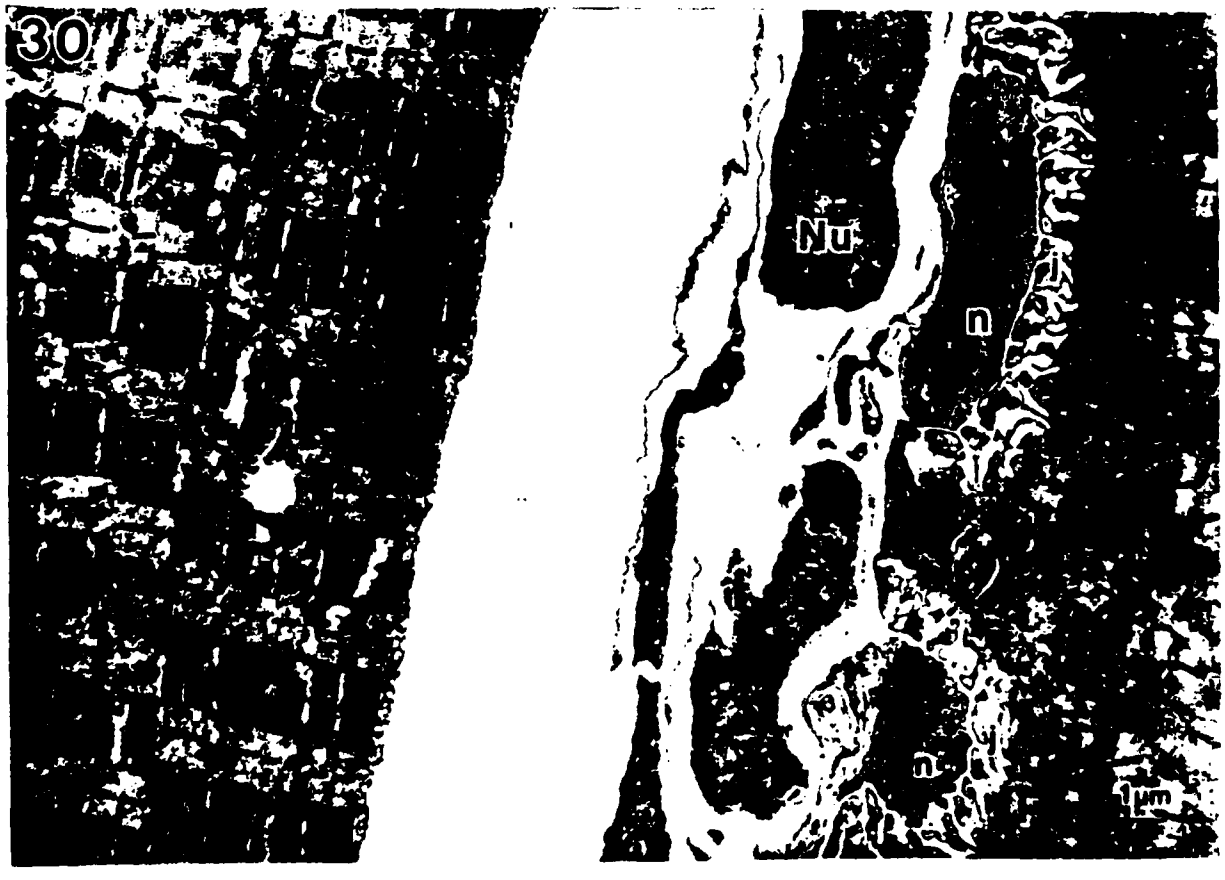



27
s
n
m
Nu
1μm

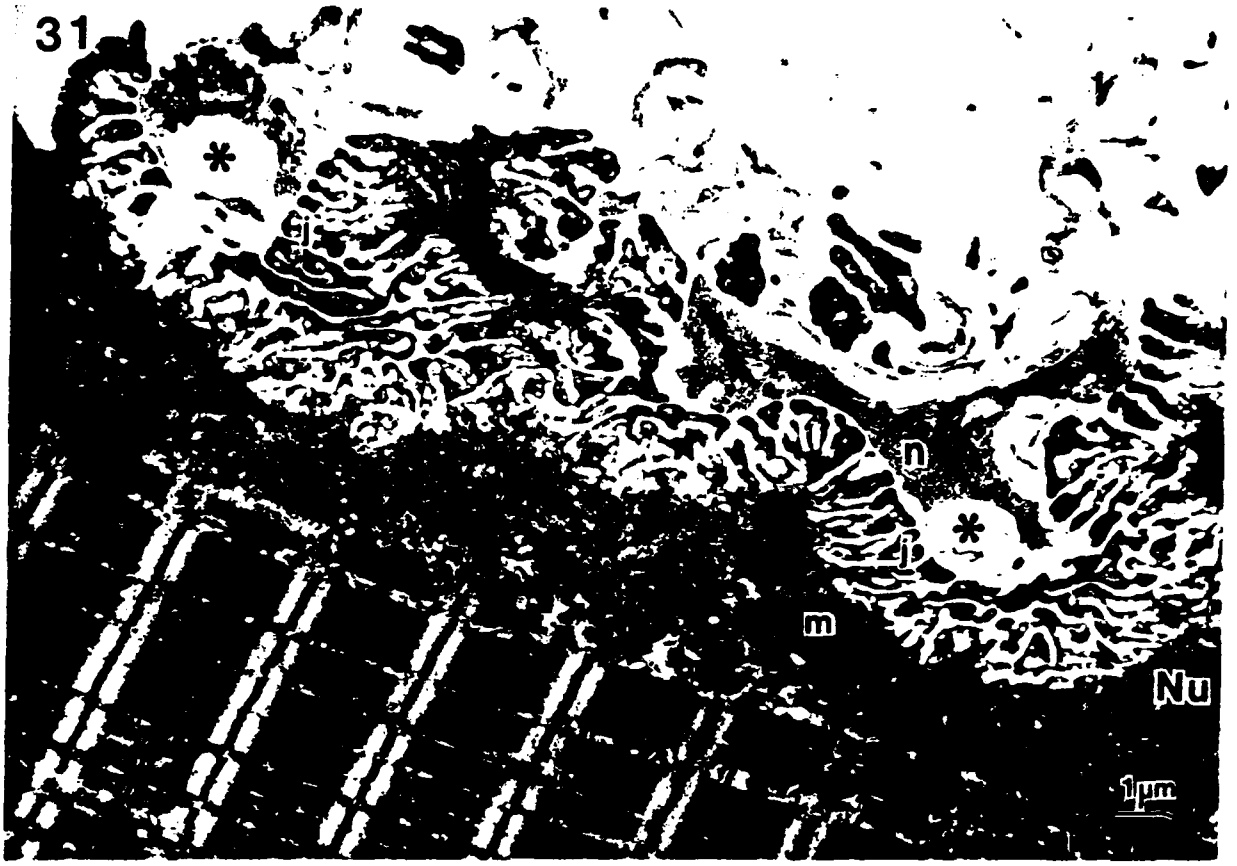

28
n
s
m
n
s
n
1μm

Figures 29 and 30. Diaphragm NMJs exposed by osmotic minipump to 20 mg of pyridostigmine over a 14 day period illustrate the potential of repair processess. The NMJ in Fig. 29 reflects damage present at 14 days exposure. The nerve terminal shows large regions of separation from postsynaptic folds (*) with concomitant Schwann cell (s) intervention in the synaptic cleft. Postsynaptically, the sarcomeres are much shorter than rest length sarcomeres from nonjunctional areas (not shown) of the same muscle fiber. The NMJ in Fig. 30 received the same drug exposure but remained drug-free for an additional 60 days. No obvious signs of damage are present.


29
n
s
*
m
j
Nu
1µm

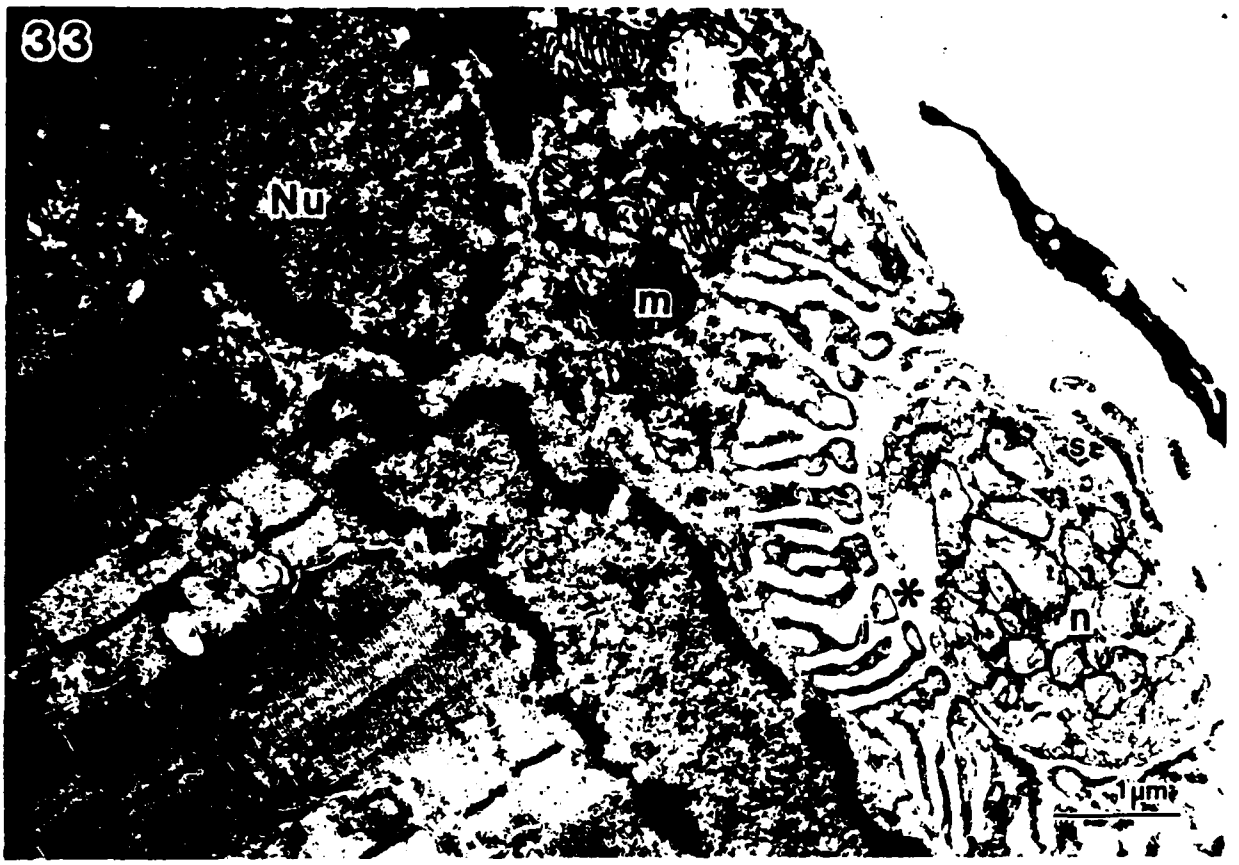

30
Nu
n
n
1µm

Figures 31 and 32. NMJs from two soleus fibers which received a 14 day subacute exposure of 20 mg of pyridostigmine reflect considerable presynaptic damage. The nerve terminal branches in Fig. 18 are widely separated from the junctional fold crests in several places (*). In Fig. 19 pre- and postsynaptic separation is due to the presence of Schwann cell processes in the synaptic cleft. Note the irregular margins of the postsynaptic nucleus.


31
n
j
m
Nu
1µm


32
n
j
Nu
1µm

Figures 33 and 34. These EDL NMJs exhibit the effects of a 14 day exposure to 20 mg of pyridostigmine via osmotic minipump. On the 14th day, the NMJ in Fig. 33 possesses moderately altered pre- and postsynaptic mitochondria (m). One region of nerve terminal is notably separated (*) from the postsynaptic folds. Following 60 days of recovery from the drug exposure, the NMJ in Fig. 34 possesses morphology similar to that seen in control NMJs. However, the nucleus has an extremely irregular margin and is associated with an abnormal number of Golgi.


33
Nu
m
Sp
*
n
1 µm


34
Nu
n
Nu
Gj
m
1 µm

Figure 35. Following 90 days recovery from a single injection of 0.036 mg/kg pyridostigmine, NMJ morphology is similar to that observed in control preparations. Occasional localized separations of pre- and postsynaptic components may be similar in number to those observed in control NMJs. Some postsynaptic nuclei (n) exhibit highly irregular margins.

35

Figure 36. Following 90 days recovery from a 14 day exposure to 20 mg of pyridostigmine by osmotic minipump (10 mg/ml), most NMJ components appear normal. Some regions of localized separation of pre- and postsynaptic components are present.


3.6

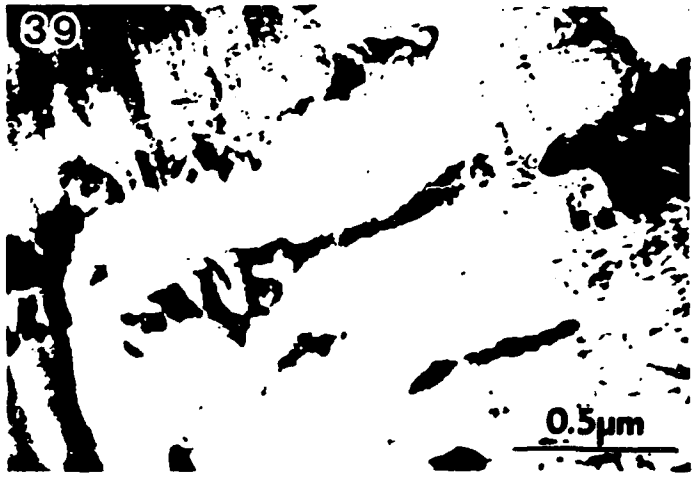
Figure 37. Side (above) and top views of specimen support designed to allow rapid freezing and cryosectioning of neuromuscular junction regions of small bundles of muscle fibers. The arrow points to the region of optimum freezing. The ends of the muscle bundle are secured in silastic-filled wells (arrowheads) by small pins.

37

Figure 38. Light micrograph of cross section through a frozen muscle bundle. Note the region of optimum freezing at arrow. Moving to either side, away from the region of impact with the liquid-nitrogen-cooled copper block, spaces of increasing size can be observed. These are due to ice crystal formation. Ice crystals increase with size with increasing distance from the point of contact.

Figure 39. In this unstained, low contrast transmission EM image of a frozen section, the mitochondria (m) are apparent and the striated pattern of the myofibrils is faintly discernable.


38


39
0.5µm